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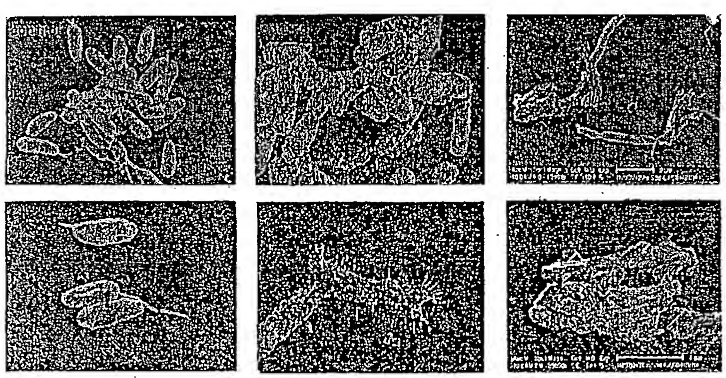
(54) Title: RECOMBINANT PROTEINS AND PEPTIDES FOR ENDOTOXIN BIOSENSORS, ENDOTOXIN REMOVAL, AND ANTI-MICROBIAL AND ANTI-ENDOTOXIN THERAPEUTICS

Scanning EM to show how Sushi peptides kill Bacteria



P. aeruginosa *K. pneumoniae* *H. pylori*

Sushi peptides puncture holes (*P. aeruginosa* & *K. pneumoniae*) into or "de-coat" (*H. pylori*) these multiple antibiotic-resistant strains of bacteria.

(57) Abstract: Recombinant fragments of Factor C are disclosed. These proteins and peptides show great potency in recognizing, binding to, neutralising, and removing endotoxin. These molecules can thus be used for anti-microbial, anti-endotoxin, and anti-sepsis therapy. SSCrFCES is a 38 kDa protein representing the LPS-binding domain of Factor C. The ability of SSCrFCES to bind lipid A was analyzed using an ELISA-based assay as well as surface plasmon resonance. Surface plasmon resonance similarly carried out for SSCrFC-sushi-1,2,3-GFP, SSCrFC-sushi-1GFP, and SSCrFC-sushi-3GFP confirmed their superior affinity for endotoxin. The 50 % endotoxin-neutralizing concentration of SSCrFCES against 200 EU of endotoxin is 0.069 µM, suggesting that SSCrFCES is an effective inhibitor of LAL coagulation cascade. Although partially attenuated by human serum, as low as 1 µM of SSCrFCES inhibits the LPS-induced secretion of hTNF-α and hIL-8 THP-1 and human peripheral blood mononuclear cells with a potency more superior than polymyxin B. SSCrFCES is non-cytotoxic, with a clearance rate of 4.7 ml/minute. The LD_{90} of SSCrFCES for LPS lethality in mice is achieved at 2 µM. These results demonstrate the endotoxin-neutralizing capability of SSCrFCES *in vitro* and *in vivo*, as well as its potential for use in the treatment of endotoxin-induced septic shock. Also embodied in this application is the use of the sushi peptides and their mutant derivatives as potent antimicrobials. Further embodied in this application is the use of sushi peptides or sushi recombinant proteins to remove endotoxin from liquids.

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RECOMBINANT PROTEINS AND PEPTIDES FOR ENDOTOXIN BIOSENSORS, ENDOTOXIN REMOVAL, AND ANTI-MICROBIAL & ANTI-ENDOTOXIN THERAPEUTICS

FIELD OF THE INVENTION

The present invention relates to the use of recombinant polypeptides and synthetic peptides derived from a horseshoe crab Factor C as well as computationally designed peptide analogues, all of which have endotoxin-binding domain(s). The recombinant proteins may be expressed from insect cell clones, either as is or as fusion proteins, e.g. with green fluorescent protein (GFP). The extreme sensitivity of the present recombinant Factor C to LPS, with its unique LPS-binding domains which have unsurpassed binding affinity for LPS, may be exploited in accordance with the present invention for anti-endotoxin and anti-microbial therapeutics as well as for the tracing, detection, and removal of LPS or gram-negative bacteria. The present invention also relates to a method for treating bacterial infection of a subject by inducing bacteriostasis by administration of a recombinant Factor C protein.

BACKGROUND OF THE INVENTION

Endotoxin, also known as lipopolysaccharide (LPS), is an integral component of the gram-negative bacterial cell membrane and is responsible for many, if not all, of the toxic effects that occur during gram-negative bacterial sepsis (1). LPS is a mixture of glycolipids consisting of a variable polysaccharide domain covalently bound to a conserved glucosamine-based phospholipid known as lipid A. LPS directly stimulates host monocytes and macrophages to secrete a wide array of inflammatory cytokines that include tumor necrosis factor-α (TNF-α), interleukins-1 (IL-1), and interleukin-8 (IL-8) (2). Excessive release of these cytokines by host macrophages almost assuredly contributes to organ failure and death that occur after episodes of gram-negative bacterial sepsis (3). The proinflammatory bioactivities exhibited by LPS typically reside in the lipid A moiety (4).

LPS from gram-negative bacteria induces the amoebocytes of horseshoe crabs to aggregate and degranulate. Presumably, the LPS-induced coagulation cascade represents an important defense mechanism used by horseshoe crabs against invasion by gram-negative bacteria (5). The amoebocyte lysate constituted as the Limulus amoebocyte lysate (LAL) test has been used for decades as a tool for detecting trace concentrations of LPS in solution (6,7). The molecular mechanism of coagulation in horseshoe crab has been established and it involves a protease cascade. This cascade is based on 3 kinds of serine protease zymogens, Factor C, Factor B, proclotting enzyme, and one clottable protein, coagulogen (8). Being the initial activator of the clotting cascade, Factor C functions as a biosensor that responds to LPS.

Despite advances in antimicrobial therapy, septic shock and other clinical complications due to Gram-negative bacterial infections continue to pose a major problem. Endotoxin or lipopolysaccharide (LPS) present on the cell wall of Gram-negative bacteria (GNB) plays an important role in the pathophysiology of these infections. It does so by mediating toxicity and also mediating release of factors like tumor necrosis factor and interleukins (40), and also by forming a rigid shield around the bacteria protecting them from the effects of antibiotics. Therefore, the detection and/or removal of LPS from the bloodstream or any parenteral solution may aid in the prevention of the inflammatory and pyrogenic effects of LPS. The lipid A component of LPS plays the most important biological role; lipid A gives rise to all the ill effects elicited by endotoxin.

A number of LPS-binding proteins have been identified. Among them are the LPS binding protein, LBP (41), and bactericidal permeability increasing protein, BPI (18,42). LBP, a 60 kDa mammalian serum protein, has a binding site with a high degree of specificity for lipid A (43). BPI, a 55 kDa protein found in human neutrophils, is capable of binding to the toxic lipid A moiety of LPS resulting in neutralization of the endotoxin (18,42,44,45).

The circulating amoebocytes of the horseshoe crab contain an array of proteins that are capable of binding and neutralizing LPS. The Limulus antilipopolysaccharide factor, LALF, an 11.8 kDa LPS-binding peptide, has been

identified in the amebocytes of horseshoe crabs *Limulus polyhemus* and *Tachypleus tridentatus*. LALF has subsequently been isolated and characterized (46-49). Purified LALF has been shown to bind LPS and exhibit endotoxin neutralization (50,19,51,52). Two other LPS-binding proteins from horseshoe crab hemocytes are tachyplesin (53,54) and big defensin (55).

Factor C is a serine protease zymogen. It is the key enzyme in the *C. rotundicauda* amoebocyte lysate (CAL) that is activated by LPS to initiate the coagulation cascade (56-58). Factor C activity is the basis of a very sensitive assay for femtogram levels of endotoxin used in the quality control of pharmaceutical products (59). The importance of Factor C in the detection of endotoxin has thus led to the expression of recombinant Factor C, rFC (12,60,61,73-38), as an alternative source that should alleviate the batch-to-batch and seasonal variation in the sensitivity of detection of endotoxin which is a recognized drawback with conventional amoebocyte lysate (59-61).

SUMMARY OF THE INVENTION

Since Factor C can be activated by femtograms of LPS, it is thought that Factor C has an LPS-binding region that exhibits exceptionally high affinity for LPS. Consequently, this LPS-binding domain can be utilized to detect and remove pyrogenic contaminants in pharmaceutical products intended for parenteral administration as well as for in vivo immunohistochemical determination of endotoxin localization (9).

The LPS-binding property of Factor C resides in the amino-terminal region spanning 333 amino acids. This short region constitutes a signal peptide, a cysteine-rich region, followed by epidermal growth factor-like domain and finally 3 sushi domains. High LPS affinity, comparable to the native Factor C, requires the correct formation of 9 disulfide bonds (16). This obstacle is compounded by the presence of a cysteine-rich region. Here, for the first time, we report the expression and secretion of a functional LPS-binding domain of *C. rotundicauda* Factor C (SSCrFCES) via a novel secretory signal. The secretory signal is disclosed in US Patent Application No. 09/426,776, filed October 26, 1999. The entire disclosures of

09/426,776 and of the provisional application upon which it is based, 60/106,426, are hereby expressly incorporated by reference.

Homologous Factor C zymogen cDNAs have been cloned from one of the four extant species of horseshoe crab, *Carcinoscorpius rotundicauda* (CrFC) (10). Initial attempts to express CrFC and its truncated forms in *E. coli* resulted in a non-active enzyme (11). Subsequently, CrFC was cloned and expressed in *Saccharomyces cerevisiae* and a methylotropic yeast, *Pichia pastoris*. However, neither the Factor C nor the *Saccharomyces cerevisiae* α mating factor signal sequences were capable of directing secretion of the recombinant protein into the culture media for easier purification (12). Full-length CrFC expressed in yeast was not enzymatically active although it retained endotoxin-binding properties (13).

Expression in a baculoviral system (US Patent Application No. 09/081,767, filed May 21, 1998) yielded recombinant Factor C (rFC) with LPS-inducible enzyme activity. The entire disclosures of 09/081,767 and of the provisional application upon which it is based, 60/058,816, are hereby expressly incorporated by reference. The rFC has extremely high sensitivity to trace levels of LPS (<0.005 EU/ml). Before these experiments, the LPS-binding domain of Factor C exhibiting high affinity for LPS was never before successfully expressed in a heterologous host. The difficulty in doing so was largely due to its highly complex mosaic structure. While many highly disulfide-bonded proteins, like epidermal growth factor (14) and secreted acetylcholinesterase (15), were successfully expressed, few display the kind of complexity posed by the Factor C LPS-binding domain.

A form of SSCrFCES was secreted in accordance with the present invention and was purified to homogeneity. The biological functions of the recombinant SSCrFCES were assessed by measuring the ability of the SSCrFCES to bind lipid A using an ELISA-based lipid A binding assay as well as surface plasmon resonance interaction. Other subfragments containing the LPS-binding domain(s) -- e.g., SSCrFCsushi-1,2,3-GFP, SSCrFCsushi-1-GFP, SSCrFCsushi-3-GFP (fusion constructs with green fluorescent protein, GFP) -- as well as synthetic peptides, e.g., sushi-1 (S1), sushi-1Δ (S1Δ), sushi-3 (S3), and sushi-3Δ (S3Δ), each of 34 mer length, and

designed variant forms of peptides bearing BHBHB and/or BHPHB (where B=basic, H=hydrophobic, P=polar amino acids) -- also show strong affinity for endotoxin.

The ability of these proteins and peptides to mediate inhibition of endotoxin-induced *Limulus* amoebocyte lysate (LAL) coagulation was measured with a sensitive LAL Kinetic-QCL assay. The SSCrFCES protein and peptides were also tested for their ability to suppress LPS-induced cytokines (TNF-α and IL-8) produced by THP-1 and normal human peripheral blood mononuclear cells (hPBMC). SSCrFCES and the peptides were non-cytotoxic. SSCrFCES has a clearance rate of 4.7 ml/min. We also show that low doses of SSCrFCES protein and the synthetic peptides protect galactosamine-sensitized mice from LPS-induced lethality. The peptides have strong antimicrobial potencies and can therefore be used as potent therapeutics.

The present invention thus includes treating bacterial infections by administration of proteins or peptides that will bind to endotoxins, especially endotoxins produced by gram-negative bacteria, to an infected subject. The binding is apparently mediated by the lipid A component of the endotoxin. The administered protein/peptide: induces bacteriostasis (that is, inhibition of bacterial proliferation) in the subject; incurs anti-endotoxic effects in vitro and in vivo (protecting mice from lethality due to endotoxaemis); causes microbicidal action aginst Gram negative bacteria (e.g., *E. coli*, *K. pneumoniae*, *S. typhimurium*, *P. aeruginosa*, *V. parahaemolytica*, *A. hydrophila*, *H. pylori*, and *S. somei*) at very high therapeutic index.

Also embodied in this invention is the use of Factor C either as a whole protein or fragments/parts thereof, or as fusion to GFP, as a biosensor for LPS or live bacteria. Further embodiend in this invention is the use of these proteins/parts thereof for LPS-removal.

A preferred embodiment of this aspect of the invention is one wherein recombinant Factor C is the administered protein. The recombinant Factor C can be a full-length Factor C protein, or any portion thereof that retains the activity of binding to lipid A. It is not necessary that the Factor C retain its serine protease enzymatic activity for the protein to be effective in the method of the invention. It may in fact be beneficial if the serine protease activity is absent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1(A). Coomassie brillant blue-stained 12% reducing SDS-PAGE profile of crude and purified SSCrFCES. The recombinant protein, SSCrFCES, was effectively secreted into the culture medium of S2 cells and identified as a 38 kDa protein band. Purification using ISOPrime™ resulted in an isoelectrically homogenous SSCrFCES.

Figure 1(B). Immunoblotting analysis was perfomed with INDIA™ His-HRP antibody and visualized using SuperSignal™ Chemiluminescence. A specific 38 kDa band, in close agreement to calculated SSCrFCES size, was identified as the only secreted and purified protein harbouring a poly-histidine tag. Exposure time, using Biomax™ film (Kodak), was limited to 5 sec. Lanes are identified as follows: 1, Low-Molecular Weight marker (Pharmacia); 2, control medium (30 μg); 3, crude SSCrFCES medium (30 μg); 4, Affinity purified SSCrFCES (1 μg); 5, ISOprime™ purified SSCrFCES (1 μg)

Figure 2(A). SSCrFCES displayed a biphasic binding profile to lipid A measured by an ELISA-based assay. Three different concentrations of lipid A were coated overnight onto Polysorp™ plates (Nunc). Varying concentrations of SSCrFCES were allowed to interact with the immobilized lipid A. The amount of bound SSCrFCES was determined by rabbit anti-SSCrFCES IgG and quantitated by ABTS substrate. The O.D.$_{405nm}$ of the samples and reference wavelength at 490nm were determined using a microtiter plate reader. The biphasic response is indicative of multiple binding sites for lipid A.

Figure 2(B). SSCrFCES binds to lipid A at a stoichiometry of ~3 lipid A molecules per SSCrFCES. A plot of the molar ratio of bound SSCrFCES to immobilized lipid A, gave a value of 0.37 at saturation. This means that each SSCrFCES molecule has the ability to bind ~3 lipid A molecules.

Figure 2(C). A Hill's plot showing Hill's coefficient, determined by the slope of the straight line obtained from plotting that data according to the Hill's equation, is 2.2. This indicates that SSCrFCES exhibited positive cooperativity in lipid A binding.

Figure 3(A). A surface plasmon resonance (SPR) sensogram depicting the interaction of SSCrFCES, with immobilized lipid A. 800ng/100 μl of SSCrFCES was

After 6 h of stimulation, the culture medium was assayed for TNF-α. The decrease in TNF-α were expressed as percentage of control (LPS only). Complete inhibition of TNF-α was achieved using 1 μM of SSCrFCES.

Figure 5(B). SSCrFCES inhibits LPS-induced hIL-8 secretion from THP-1 in a dose-dependent manner. PMA-treated THP-1 cells were treated with 100 ng/ml of *E. coli* 055:B5 LPS which was preincubated with varying concentrations of SSCrFCES. After 6 h of stimulation, the culture medium was assayed for IL-8. The decrease in IL-8 secretion was expressed as percentage of control (LPS only). 95% inhibition of IL-8 secretions were achieved using 1 μM of SSCrFCES.

Figure 6(A). The ability of SSCrFCES to inhibit LPS-stimulated TNF-α secretion from PBMC cells. In the absence of human serum, addition of only 8.5 nM of SSCrFCES caused 50% inhibition of TNF-α response to 10 ng/ml LPS. SSCrFCES pre-incubated with 10% human serum required 17-fold more protein to achieve 50% inhibition. The attenuation can be minimized if the SSCrFCES was mixed with endotoxin 5 min before the addition of serum, thus requiring only 4-fold more SSCrFCES for 50% inhibition of cytokine release.

Figure 6(B). The ability of SSCrFCES to inhibit LPS-stimulated IL-8 secretion from PBMC cells. In the absence of human serum, addition of only 8.5 nM of SSCrFCES caused 50% inhibition of IL-8 response to 10 ng/ml LPS. SSCrFCES pre-incubated with 10% human serum required 17-fold more protein to achieve 50% inhibition. The attenuation can be minimized if the SSCrFCES was mixed with endotoxin 5 min before the addition of serum, thus requiring only 4-fold more SSCrFCES for 50% inhibition of cytokine release.

Figure 6(C). The ability of synthetic peptides to suppress LPS-induced TNF-α.

Figure 7. SSCrFCES is not cytotoxic to mammalian cells. At the highest concentration of 4 mg/ml or 109 μM, only 20% cell lysis was observed.

Figure 8. Pharmacokinetic analysis of SSCrFCES shows that clearance rate of biotin-labeled SSCrFCES in C57BL/6J mice is 4.7 ml/min.

Figure 9(A). SSCrFCES protects C57BL/6J mice against LPS-induced lethality. 100% LPS-induced lethality was achieved using 2.0 ng of *E. coli* 055:B5 within 7 h. The percentage of survival was increased to >90% when 2 and 4 μM of SSCrFCES

were injected i.v. 10 min after LPS challenge. Kaplan-Meier analysis indicates that there is significant difference between 1μM and 2 μM of SSCrFCES (P<0.0005). No significant difference was observed between 2 μM and 4 μM of SSCrFCES.

Figure 9(B). S1, S1Δ, S3, S3Δ, and other designed variant peptides protect C57BL/6J mice against LPS-induced lethality. 100% LPS-induced lethality was achieved using 2.0 ng of *E. coli* 055:B5 within 7 h. The synthetic peptides (25 or 75 μg) were pre-incubated with LPS for 30 min prior to i.p. injection. S1, S1Δ, and S3 conferred 20-55% decrease in LPS-induced lethality. However, S3Δ is significantly more effective in protection, where 75 ug was sufficient to confer 100 % protection.

Figure 10(A). CrFC21 (SEQ ID NO:4) showing functional domains of Factor C.

Figure 10(B). Recombinant fragments: ssCrFCES; sushi-1,2,3-EGFP; sushi 1-EGFP; and sushi-3-EGFP fusion proteins. Sushi peptides of 34 mer each (S1, S1Δ, S3, & S3Δ).

Figure 11A. Sequences of V1 and V2 peptides.

Figure 11B. Sequences of peptides featured in Table 3.

Figure 12. The microbicidal concentrations (MBC) of sushi peptides against *P. aeruginosa* (ATCC 27853). An initial density of 10^5 cfu/ml of *P. aeruginosa* was used in the assay. Doubling of the peptide concentrations in the region of 0.03-0.5 ug/ml resulted in exponential reduction of bacterial count. S1 and S3 were more effective against *P. aeruginosa* than S1Δ and S3Δ.

Figure 13. Time-dependent killing of *P. aeruginosa* ATCC 27853. An initial density of 10^9 cfu/ml of *P. aeruginosa* was used in the assay. The effect of test peptides at 0.06 ug/ml was assessed by enumerating the viable (cfu/ml) at indicated time intervals after overnight incubation. The bacterial count was exponentially reduced to achieve MBC_{90} within 7 min. By 30-40 min, the bacterial was completely eradicated.

Figure 14. Drop count plates for the killing rate assay at 0.06 ug/ml of sushi peptides in general, monitored at the indicated time intervals. Segments of the plates contain *P. aeruginosa* culture at 10-fold dilution starting from 10^{-1} to 10^{-8} from the upper quadrant in anti-clockwise direction. S3Δ peptide eradicated the bacteria at a log reduction rate.

Figure 15. Electron micrographs showing examples of how the antimicrobial peptides kill the bacteria.

Figure 16. Sushi peptides display negligible hemolytic activities. Human and rabbit erythrocytes at 0.4% were reacted separately with different doses of peptides (6-100 µg/ml). 0.4% erythrocytes lysed in 1% Triton-X was taken as 100% lysis. The negative control was 0.4% erythrocytes in pyrogen-free saline. Sushi peptides were minimally hemolytic up to concentrations of 100 µg/ml. S1, S1Δ, and S3 showed negligible haemolysis and S3Δ caused a 35% haemolysis at 100 µg/ml. Concentration of peptide to induce 50% haemolysis: S1 290 µg/ml; S1Δ 295 µg/ml; S3 160 µg/ml; and S3Δ 120 µg/ml.

Figure 17. Example of S3Δ-peptide coupled Agarose CL-6B beads bound with FITC-LPS, seen under microscope. (A) Bright field observation; (B) Beads with FITC-LPS bound, seen under UV light; (C) Bound beads after treatment with 1% DOC - no FITC-LPS left on the beads (observed under UV light).

Figure 18. A test of binding conditions of LPS to S3Δ peptide affinity beads under increasing pH and ionic strength. (A) pH of 4.0 and 5.0 (in 20 mM sodium acetate), pH 6.8 and 9.1 (20 mM Tris-HCl). All buffers were supplemented with 50 mM NaCl. (B) Different ionic strength: 20 mM Tris-HCl (pH 6.8) were supplemented with different concentrations of NaCl, except of the 0 mM point which contained pyrogen-free water as control.

Figure 19. Immunoblot showing expression of rFC (pHILD2/CrFC21; lane 1), rFCEE (pHILD2/CrFC21EE; lane 2) and rFCSN (pPIC9/CrFC26SN; lane3) in the crude supernatant. Arrows indicate the immunoreactive recombinant Factor C proteins: 132 kDa full-length rFC, 90 kDa truncated rFCEE and 89 kDa truncated rFCSN. The molecular weight markers (MW) are labeled in kDa.

Figure 20. SDS-PAGE analysis showing the protein profiles of the different preparations of rFC: crude supernatant (lane 1); $(NH_4)_2SO_4$ precipitated sample (lane 2); Biomax™-50 enriched rFC (lane 3); and Sephadex™ G-100 purified sample (lane 4). Ten micrograms of each protein sample were loaded. Arrow indicates the 132 kDa full-length rFC. The molecular weight markers (MW) are labeled in kDa.

Figures 21A and 21B. Modified Western blot to show binding of Factor C to LPS strips (Fig. 21A) and lipid A strips (Fig. 21B). Lanes 1: crude rFC; 2: $(NH_4)_2SO_4$ precipitated rFC; 3: Biomax™-50 purified rFC; 4: Sephadex™ G-100 purified rFC; 5: Biomax™-50 purified rFCEE; 6: Biomax™-50 purified rFCSN; 7: pHILD2/151 supernatant. The 7-20 kDa lipid A bands are indicated between the 2 arrows.

Figure 22A. Competitive effects of 50, 100 and 200 μg total protein of crude rFC on LPS-mediated activity of CAL Factor C enzyme activity. Dashed line illustrates the ratio of crude rFC to LPS (1000 : 1) for a percentage competition of >80%. Results are the means ± S.D. of three independent experiments.

Figure 22B. Competitive effects of 50, 100 and 200 μg Biomax™-50 enriched rFC on LPS-mediated activity of CAL Factor C enzyme activity. Dashed line illustrates the ratio of rFC to LPS (100 : 1) for a percentage competition of >80%. Results are the means ± S.D. of three independent experiments.

Figure 23. Comparison of the competitive efficiencies of full-length rFC and truncated rFCEE on LPS-mediated enzymatic activity of CAL Factor C. Each protein sample (rFC or rFCEE) was enriched by Biomax™-50 ultrafiltration, and 100 μg was used in the competition assay. The percentage competition was obtained after normalization with the background competition by rFCSN. Results are the means ± S.D. of three independent experiments.

Figure 24. Interactive binding of rFC to immobilized lipid A in a BIACORE X™ sensor. Lipid A (100 μg/ml) was immobilized on the sensor chip. The respective protein samples were flowed through and relative responses recorded in response units (RU) by the BIACORE X™ instrument. Plateaus 1A, 2A and 3A on the sensorgram represent the relative responses of Biomax™-50 enriched rFCSN, rFCEE and rFC, respectively, to immobilized lipid A. Arrows show the RU due to regeneration with 0.1M NaOH. Inset shows the net percentage RU of rFC and rFCEE to immobilized lipid A. The percentage RU of each protein sample was calculated based on the relative RU of the protein sample and that of immobilized lipid A. The net RUs of rFC and rFCEE were obtained after normalizing their relative RUs with that of rFCSN.

Figure 25. The bacteriostatic effects of Sephadex™ G-100 purified rFC on the growth of the Gram-negative bacteria: *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. typhimurium*. rFC was most efficacious against *K. pneumoniae* whereas the bacteriostatic activity against *P. aeruginosa* declined rapidly after 4 h.

Figures 26A-26E. Agglutination of *E. coli* by rFC (Fig. 8A) and rFCEE (Fig. 8B). Observations were made with a Nikon MICROPHOT™-FXA microscope (400X magnification). No agglutination was seen with rFCSN (Fig. 8C), pHILD2/151 (Fig 8D) and 0.85% saline (Fig. 8E).

Figure 27. The protective effect of 10 μg rFC purified through Sephadex™ G-100 on actinomycin D sensitized/ LPS-challenged mice. Pre-incubation of LPS with rFCSN did not confer protection of mice against the endotoxic effects of LPS. For comparison, rFC conferred 60-70% protection.

Figure 28. Line drawings of rFC (full length) and its deletion homologues, given with their corresponding start and end amino acid positions based on the CrFC21 clone (SEQ. ID. NOs. 3 and 4, US Patent No: 5,716,834). Amino acid residues are numbered as in SEQ. ID. NO. 4. rFC, rFCEE, rFCES(sushi-1,2,3), rFC(sushi-1) and rFC(sushi-3) have endotoxin-binding site(s). Sushi (⇩) domains 1, 2, and 3 denote secondary structures in Factor C, with 'sushi-like' folding patterns. rFCSN does not contain any endotoxin-binding site. The lines are not drawn to scale.

Figure 29. Binding of rFC produced in baculovirus-infected Sf9 cells to LPS from various bacteria.

Figure 30. Bacteriostasis induced by rFC produced in baculovirus-infected Sf9 cells in cultures of different Gram-negative bacteria.

Figure 31. Protection of mice from LPS lethality by administration of rFC produced in baculovirus-infected Sf9 cells.

BRIEF DESCRIPTION OF TABLES

Table 1 presents a comparison between binding affinity for lipid A of Factor C-derived sushi proteins and other LPS-binding proteins.

Table 2 presents a comparison of MBC_{50}, MBC_{90}, hemolytic activity, and cytotoxic activity of sushi and other cationic peptides on test microorganisms.

Table 3 provides indicators of LPS-binding, anti-LPS, and antimicrobial activities of Factor C and various peptides. In Table 3, column I shows affinity for LPS binding of peptide to Lipid A immobilized on an HPA chip, column II shows Hill's Coefficient – the stoichiometry of binding of the number of peptide molecules to 1 LPS molecule, column III shows Circular Dichroism (CD) analysis of peptide structures in the presence of 0.75 nM lipid A (α-H: α-helical; β: β-sheet; T: turn; R: random), column IV shows neutralization (EC_{50}) – μM of peptide needed to neutralize 50% of 200 EU/ml of LPS-induced LAL reaction, column V shows the amount of peptide needed to cause 50% suppresion of LPS-induced cytokine release (TNF-α), column VI shows mouse protection assays – 2 ng LPS pre-incubated with peptide for 30 minutes before injection into C57/BL, column VII shows cytotoxicity (cell lysis) assays – for S4-S9: EC_{50} = [peptide] to cause 50% lysis cytotoxicity, column VIII shows hemolytic activity at 100 μg peptide, and column IX shows MBC_{90} (microbicidal concentration of peptide that kills 90% of bacteria) or MIC_{90} (minimal inhibitory concentration of peptide that inhibits 90% of bacteria).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides efficient, high affinity recombinant proteins and peptides for gram-negative bacterial endotoxin. These molecules can be used, among other things, for: (a) anti-microbial, anti-endotoxin, anti-sepsis therapeutics; (b) tracing and detection or localization of gram-negative bacteria via, for example, the GFP component of SSCrFCsushi-GFP fusion proteins; and (c) development of LPS-specific affinity chromatography systems to purify endotoxin-contaminated samples or biological fluids.

The present invention lies in part in methods for treating bacteremia using proteins that bind to bacterial endotoxin as a therapeutic agent. A particularly effective protein is a recombinant Factor C protein, or any portion thereof that retains the biological activity of binding to lipid A.

cDNAs encoding Factor C proteins from *Carcinoscorpius rotundicauda* have been previously described (10,73). Recombinant Factor C from *Carcinoscorpius rotundicauda* (rCrFC) has been produced in vitro by coupled transcription/translation systems. However, the present invention resides partly in the development of in vivo systems, especially using insect cells as the host cell, for efficient production of rFC by expression of cloned DNA.

Also, the protection of rFC from activation and subsequent self-proteolysis by binding of endotoxin which may be present in solutions used in isolation of the protein is described in U.S. Patent No. 5,716,834, the entire disclosure of which is hereby incorporated by reference. Basically, dimethylsulfoxide (Me_2SO or DMSO) is added to solutions which are used during the purification process. Even greater protection of the rFC is achieved by also adding an agent effective for chelating divalent metal ions to the purification solutions.

cDNAs appropriate for expression in the presently-described system can be cDNAs encoding Factor C of any horseshoe crab. Two representative nucleotide sequences are presented as SEQ ID NO:1 and SEQ ID NO:3 (encoding the amino acid sequences of SEQ ID NOs:2 and 4). A composite DNA sequence, assembled from incomplete cDNA fragments, encoding the Factor C of Tachypleus tridentatus is disclosed by Muta et al (49).

Factor C appropriate for use in the present invention can be produced by any method typical in the art. Production of rFC in yeast host-vector systems is described in reference 75. Recombinant Factor C produced in yeast is found to lack serine protease activity, but, as shown in the working examples below, protein produced in yeast is still effective in both lipid A and endotoxin binding and in inducing bacteriostasis. Production of rFC in yeast host-vector systems is described in detail in co-pending U.S. Patent application 08/877,620. Recombinant Factor C for use in the invention can also be produced by a baculovirus host-vector system or in another suitable insect cell host-vector system, such as one for Drosophila cells. Co-pending U.S. Patent applications 09/081,767, 60/106,426 and 09/201,786 provide detailed description of production of rFC in such systems.

The endotoxin/lipid A-binding domain of Factor C lies within the amino terminal portion of the protein encompassed by rFCES; that is, the first 350 amino acids, numbered as in SEQ. ID. NO. 4. Referring to Figure 28, endotoxin/lipid A binding activity is found in the truncated rFCEE (amino acids 1-766), rFCES (amino acids 29-330), rFC(sushi-1) (amino acids 29-201) and rFC (sushi-3) (amino acids 264-330). Molecular modeling studies suggest that the contacts are made by portions of the protein lying in the cysteine-rich domain, especially amino acids 60-70, in the sushi-1 domain, especially amino acids 170-185 and in the sushi2 domain, especially amino acids 270-280. Thus, a protein having at least these three portions of Factor C, which can be joined by a random amino acid sequence or by other chemical linkage, is expected to be useful in the method of the present invention.

As noted above, naturally-occurring Factor C proteins, and rFC that is full-length and produced in baculovirus-infected or other insect cell lines, possesses a serine protease activity. That activity is activated by endotoxin or lipid A binding. It might be found that the serine protease activity of the rFC produces undesired side effects when treating a subject with rFC according to the invention. Thus, in preferred embodiments of the present invention, the serine protease activity of the rFC is inactivated, either chemically or by mutation, or the domain providing that activity is deleted from the protein.

The portion of Factor C from horseshoe crab that constitutes the serine protease domain is approximately from amino acid 760 to the carboxy terminus of the protein, numbered as in SEQ. ID. NO.:4. Furthermore, the particular amino acids that constitute the catalytic residues are His809, Asp865, and Ser966. Thus, inactivation of these residues by chemical modification or by site-specific mutation can be used to provide rFC that will bind to lipid A, but lacks serine protease activity.

Chemical modifications to inactivate serine protease activity are well-known in the art. Methods for introducing site-specific mutations into any particular polypeptide are also well-known in the art.

Colorimetric and fluorescent assays for the serine protease activity of rFC are described in detail in co-pending application 09/081,767, the entire disclosure of which is hereby incorporated by reference. These assays are appropriate for

screening mutant forms of rFC for serine protease activity. Assays for lipid A and endotoxin binding is also described in co-pending application 09/081,767 that can be used to ascertain that the serine protease-deficient mutant retains the lipid A/endotoxin binding activity required if the protein is to be used in the present invention.

"Stringent conditions" for hybridization are those that provide for hybridization of sequences having less than 15% mismatch, preferably less than 10% mismatch, most preferably 0% to 5% mismatch. Exemplary of such conditions, using probes of 50 bases or longer, are an aqueous solution of 0.9 M NaCl at 65 °C; an aqueous solution of 0.98 M NaCl, 20% formamide at 42-45 °C. The conditions will vary according to the length of the probe, its G+C content and other variables as known to the skilled practitioner (54). Exemplary wash conditions following hybridization are an aqueous solution of 0.9 M NaCl at 45-65 °C, preferably 55-65 °C. Lower salt, or addition of an organic solvent such as formamide, in the wash buffer will increase the stringency of the condition as known in the art.

A preferred hybridization condition is at 42°C in 50% formamide, 5x SSC, 1x Denhardt's solution, 20 mM phosphate buffer, pH 6.5, 50 μg/ml calf thymus DNA, 0.1% SDS. Salt and temperature conditions equivalent to the hybridization conditions employed can be calculated from the following equation:

$$T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - 0.63(\%formamide) - (600/l)$$

where l = the length of the hybrid in base pairs.

A preferred washing condition is in 1x SSC, 0.1% SDS washing solution at room temperature, followed by washing at high stringency with 0.1x SSC, 0.1% SDS at 42°C and 2x with 0.1x SSC/0.1% SDS for 15 min. each at 42°C.

Preferred versions of rFC for use in the method of the invention are those encoded by polynucleotides that will hybridize to a nucleic acid having the sequence of SEQ. ID. NO. 1 or SEQ. ID. NO. 3 under stringent conditions. Most preferred versions of rFC are those having the amino acid sequence of SEQ. ID. NO. 2 or SEQ. ID. NO. 4.

For administration to a subject for treatment of bacterial infection or to induce bacteriostasis, the rFC is formulated with pharmaceutically acceptable carriers appropriate for the route of administration. Formulation of polypeptides for administration is known in the art; the practitioner is referred, for example, to reference 79. The route of administration is not particularly limiting of the invention, but preferred routes are intraperitoneal, intravenous, and topical administration.

The proteins for administration are preferably formulated in pharmaceutical saline solutions such as 0.9% saline, phosphate buffered saline and the like. The polypeptides can be provided in lyophilized form and reconstituted for administration. The final concentration of the protein in the formulation administered is one that would provide a suitable dosage as described below.

Polypeptide therapeutic agents are known to be susceptible to degradation in the body, usually due to the action of proteolytic enzymes. Thus, the rFC administered according to the present invention might desirably be derivatized to inhibit such degradation. For example, carboxy-terminal amidation of the protein is known in the art to inhibit degradation by proteases present in serum. Particular derivations of proteins to improve their resistance to degradation in vivo and methods for accomplishing them are well-known in the art.

The dosage to be administered will of course be tailored to the particular form of rFC administered and the route of administration. Tailoring of dosage is considered within the skill of the routine practitioner. A dosage within the range 0.01 to 3 mg/kg body weight is acceptable; preferably the dosage will be within the range of 0.1 to 3 mg/kg, most preferably in the range of 0.3 to 0.4 mg/kg.

Doses may be administered either by bolus or by infusion. The particular rate of administration will be determined partly by the half-life of the protein in the body, which will be influenced by the particular structure of the protein and also by the route of administration. Assessment of pharmacokinetics necessary to determine the precise rate and dosage of the particular protein to be administered is considered within the skill of the practitioner.

For topical administration, rFC or polypeptides or recombinant polypeptides (rPP) in combination with oil and water emulsions at a final concentration of ≤

0.01% may be used to form topical creams/lotions/ointments. These preparations can be applied for treatment against bacterial infection of the skin, for instance, secondary burn patients (against *Pseudomonas aeruginosa*) or cellulitis (against *Staphlylococcus aureus*). The rFC or polypeptides of rPP can also be used in cosmetic, skin, or hair preparations as antimicrobial preservatives, either alone or in combination with conventional preservatives, to prevent or control the growth of bacteria, yeast, and mold.

The following exemplary embodiments of the invention serve to illustrate the invention. The examples are not to be considered limiting of the scope of the invention, which is defined only by the claims following.

Example 1: Purification of stably expressed and secreted recombinant SSCrFCES

Stable cell lines of Drosophila S2 clones expressing SSCrFCES (US Patent Application No. 09/426,776) were routinely cultured in serum-free DES Expression medium and maintained at 25°C in a humidified incubator.

(a) Purification of SSCrFCES using a TALON column

The medium containing SSCrFCES was initially concentrated and desalted via 3 rounds of ultrafiltration using a 10 kDa cutoff membrane in an Amicon stirred cell (Millipore). Affinity chromatography purification under denaturing conditions yielded a 38 kDa protein of interest, in addition to a 67 kDa protein. Western blot analysis indicated that the 67 kDa protein does not contain the carboxyl poly-His tag. Thus this larger protein is likely due to non-specific adsorption to the resin.

(b) Purification of SSCrFCES by Preparative Isoelectric Membrane Electrophoresis

Typically, 2 liters of conditioned medium were initially subjected to successive ultrafiltration using a 100 kDa and 10 kDa molecular weight cutoff with the Pellicon system (Millipore). The medium was concentrated seven-fold. The enriched SSCrFCES was purified to isoelectric homogeneity using Preparative Isoelectric Membrane Electrophoresis (Hoefer IsoPrime™, Pharmacia). The pI of the SSCrFCES was determined to be 7.1 at 4°C. A set of four membranes were made, with pHs of 6.5, 7.0, 7.25, and 7.5. The concentration of acrylamido buffers used for the membranes were calculated based on information in Righetti and Giaffreda (17).

The four membranes were assembled in order, from acidic to basic, to delimit five chambers. Each sample reservoir vessel was filled with 30 ml of pyrogen-free water and pre-run at 4°C at 4 Watts constant power (3000 V limiting, 20 mA maximum) for two hours.

After removing the pre-run water, the protein sample was placed in sample reservoir vessel corresponding to the chamber delimited by pH 7.0 and 7.25. The IsoPrime was conducted under the same conditions for 3-4 days without detrimental effect on the protein, and the content from each chamber was analyzed on a 12% SDS-PAGE. The scheme reported here has been found to be reproducible in our laboratory throughout the course of approximately two years. The overall recovery of SSCrFCES binding capacity is nearly 90%. This is attributable to its extreme stability conferred by the presence of 9 disulfide bonds.

(c) Analysis of the purified SSCrFCES

The distribution of the protein was identified using Chemiluminescent Western blot. SDS-PAGE analysis of Drosophila cells transformed with the recombinant vector is shown in Figure 1A. The Western blot revealed the presence of a protein with an apparent molecular weight of ~38 kDa (Figure 1B). SSCrFCES in medium represented > 90% of the total recombinant protein expression. When stable cell line was cultured in serum-free medium without hygromycin for a week in a 1L-Bellco spinner flask, a typical yield ~1.6 mg/L of SSCrFCES was achieved.

The presence of SSCrFCES in the culture medium thus contributes to the ease of batch-continuous culture and purification. Most significantly, SSCrFCES expressed and secreted from insect cells was biologically active.

Example 2 : ELISA-based Lipid A binding assay

A Polysorp™ 96-well plate (Nunc) was first coated with 100 μl per well of various concentrations of lipid A diluted in pyrogen-free PBS. The plate was sealed and allowed to incubate overnight at room temperature. The wells were aspirated and washed 6 times with 200 μl per wash solution (PBS containing 0.01% Tween-20 and 0.01% thimerosal). Blocking of unoccupied sites was achieved using wash solution containing 0.2% BSA for 1 hour at room temperature. Subsequently,

blocking solution was removed and the wells washed as described above. Varying concentrations of SSCrFCES were allowed to interact with bound lipid A at room temperature for 2 hours.

Bound SSCrFCES was detected by sequential incubation with rabbit anti-SSCrFCES antibody (1:1000 dilution) and goat anti-rabbit antibody conjugated with HRP (1:2000 dilution) (Dako). Incubation with each antibody was for 1 h at 37°C with washing between incubations as described above. In the final step, 100 μl of peroxide substrate ABTS (Boehringer Mannheim) was added. Using a microtiter plate reader, the absorbance of the samples was determined at 405nm with reference wavelength at 490nm. The values were correlated to the amount of LPS bound and SSCrFCES present. Quantitation of SSCrFCES was achieved from a standard curve derived by immobilizing known amount of purified SSCrFCES onto a Maxisorp plate.

Results from the ELISA-based lipid A binding assay displayed a biphasic curve (Figure 2A). Unlike other LPS-binding proteins (18-21), SSCrFCES has multiple binding sites for the ligand. SSCrFCES binds co-operatively to lipid A with a stoichiometry of one SSCrFCES to ~3 lipid A molecules at saturation (Figure 2B). Scatchard plots of the binding data are very convex, indicating that the binding of SSCrFCES to lipid A is highly cooperative, being comparable to haemoglobin for oxygen (22). This is confirmed by the slope of the line obtained from plotting the data (Figure 2C) according to the Hill's equation (23), which gave a coefficient of 2.2. While bactericidal/permeability-increasing protein (BPI) (18) was reported to bind > 1 lipid A molecule, it was not reported to exhibit cooperativity in binding. This homotropic cooperativity for binding to lipid A is thus novel and unique to SSCrFCES.

The presence of multiple lipid A binding sites that showed cooperativity assuredly confirm the LPS-binding domain of Factor C, as well as full-length Factor C, to be the best candidate for removal and detection of endotoxin in solution, and supports its use as an anti-endotoxin therapeutic. Cooperative binding also contributed to Factor C's ability to detect sub-picogram level of endotoxin (US Patent Application No. 09/081,767) as well as a competitive binding advantage over Limulus Anti-LPS Binding Factor (LALF).

Retrospectively, the degranulation of amoebocytes in the presence of LPS would release a battery of anti-bacterial/LPS binding factors e.g. LALF, thus significantly reducing the amount of free LPS. Nonetheless, Factor C is capable of capturing trace LPS to activate the coagulation cascade. Such capability is attributed to its homotropic cooperativity as demonstrated by SSCrFCES, that is to say, its LPS-binding domain.

Example 3: Surface Plasmon Resonance (SPR) studies on biospecific binding kinetics between lipid A and: CrFCES; SSCrFCsushi-1,2,3-GFP; SSCrFCsushi-1-GFP; SSCrFCsushi-3-GFP; and synthetic peptides

Recognition of lipid A by the abovenamed secreted recombinant proteins and peptides was performed with a BIAcore X™ biosensor instrument and an HPA sensor chip. Briefly, lipid A at 0.5 mg/ml in PBS was immobilized to a HPA sensor chip (Pharmacia) according to the manufacturer's specification. In all experiments, pyrogen-free PBS was used as the running buffer at a flow rate of 10 μl/min.

With purified SSCrFCES, 4 μg/ml was injected into the flow cell at a rate of 10 μl/min, and the binding response was measured as a function of time. Following injection of SSCrFCES, a solution of INDIA™ HisProbe™-HRP antibody, diluted in PBS to 400 μg/ml, was also injected to cause a shift in SPR in order to further confirm that SSCrFCES binds to lipid A. For regeneration, 100 mM of NaOH solution was injected for 5 minutes. Similar lipid A binding analysis was carried out with SSCrFCsushi-GFP fusion proteins.

Figure 3A shows that injection of 400 ng/100 ul of SSCrFCES over immobilized lipid A resulted in an increase of ~200 relative response unit. This represents a 92% saturation of lipid A. Subsequently, injection of antibody (INDIA™ His-HRP Ab) against the poly-His tag of SSCrFCES resulted in a further increase of relative response unit. The binding of INDIA™ His-HRP Ab further confirms that only SSCrFCES was bound to the immobilized lipid A.

Figures 3 B, 3C, and 3D show SPR (in response units) of the realtime binding interactions between SSCrFCsushi-1,2,3, SSCrFCsushi-1, and SSCrFCsushi-3-GFP fusion proteins, respectively, to the immobilized lipid A on the biochip. Figure 3E

shows the same binding interaction analysis of four examples of synthetic peptides derived from sushi-1 and sushi-3 of Factor C.

Example 4: SSCrFCES and synthetic peptides are potent anti-endotoxin molecules – (inhibition of endotoxin-induced LAL reaction)

The Limulus Kinetic-QCL is a quantitative, kinetic assay for the detection of gram-negative bacterial endotoxin. This assay utilizes the initial part of LAL endotoxin reaction to activate an enzyme, which in turn releases p-nitroaniline from a synthetic substrate, producing a yellow color. The time required before the appearance of a yellow color is inversely proportional to the amount of endotoxin present. Throughout the assay, the absorbance at 405 nm of each well of the microplate was monitored. Using the initial absorbance reading of each well as its own blank, the time required for the absorbance to increase 0.200 absorbance units were calculated as Reaction Time. The 50% endotoxin-neutralizing concentration (ENC_{50}) reflects the potency of SSCrFCES or the synthetic peptides; a low ENC_{50} indicates high anti-endotoxin potency.

Briefly, 25 µl of endotoxin solution (LPS, *E.coli* 055:B5) at 200 EU/ml was mixed with an equal volume of SSCrFCES at 1 µM, in a series of 2-fold dilutions in LAL reagent water in disposable endotoxin-free glass dilution tubes (BioWhittaker) and incubated at 37°C for one hour. The reaction mixtures were each diluted 1000-fold with LAL reagent water. The endotoxin activity was then quantified with Limulus Kinetic-QCL. One hundred µl of the diluted test mixture was carefully dispensed into the appropriate wells of an endotoxin-free microtitre plate (Costar). The plate was then pre-incubated for >10 minutes in a temperature-controlled ELISA plate reader. Near the end of the pre-incubation period, 100 µl of freshly reconstituted Kinetic-QCL reagent was dispensed into the wells using an 8-channel multipipettor. The absorbance at 405 nm of each well of the microtitre plate was monitored at time intervals of 5 minutes over a period of 2 hours. A 5 second automix was activated prior to reading. In the Limulus Kinetic-QCL, the assay was activated by 0.005 EU/ml of endotoxin.

The high sensitivity of the assay allowed for very low levels of endotoxin to be detected. Following incubation of endotoxin with SSCrFCES, a 1000-fold dilution was introduced to eliminate any potential effects of the SSCrFCES on the LAL enzyme system. A sigmoidal curve is usually expected between relative reaction time and the logarithmic concentration of the SSCrFCES. The best fit curve was derived using SigmaPlot and the concentration corresponding to 50% relative increase in reaction time was designated ENC_{50}. The mean values were obtained from 3 independent experiments.

The time that is required before the appearance of a yellow color (Reaction Time) is inversely proportional to the amount of endotoxin present. A low ENC_{50} indicates high potency of endotoxin neutralization. The ENC_{50} is taken as the concentration of SSCrFCES that reduces the mean reaction time by 50%. A sigmoidal curve was obtained between relative reaction time and the logarithmic concentration of SSCrFCES (Figure 4). ENC_{50} of SSCrFCES was determined to be 0.069 ± 0.014 μM. Comparatively, this value is 28- and 7.5-fold less than ENC_{50} of polymyxin B and LF-33 (33-mer peptide derived from lactoferrin) (24), respectively. This shows that on a molar basis, much less SSCrFCES is required to neutralize the same amount of LPS. Consequently, it also indicates that SSCrFCES is a potent anti-pyrogenic recombinant protein.

The ENC_{50} of the synthetic sushi peptides were comparable to other reported peptides, e.g.: S1=2.25 μM; S1Δ =0.875 μM; S3=1 μM; A3 =0.625 μM. For the designed peptides, the ENC_{50} values were: V1=0.47 μM and V2=0.89 μM.

Hill's plot for the interaction between synthetic peptides and lipid A shows that S1 exhibited high positive co-operativity of $n = 2.42$, indicating that more than two S1 peptides interact with one LPS molecule.

<u>Example 5: The anti-sepsis activities of SSCrFCES and synthetic peptides: inhibition of the LPS-induced TNF-α and IL-8 by (a) THP-1 cells (b) human peripheral blood mononuclear cells (PBMC)</u>

During gram-negative bacterial septicaemia, the high concentration of LPS in the blood leads to multiple organ failure syndromes. These adverse effects are dependent on the generation of endogenous mediators. A multitude of mediators

have been implicated, including arachidonic acid metabolites, PAF, cytokines such as TNF-α, interferons, and various interleukins (e.g. IL-1, IL-8, etc.), reactive oxygen metabolites, and components of the coagulation cascade (1-3). Consequently, the biological potential of SSCrFCES to bind and neutralize LPS-stimulated production of cytokines in human promonomyelocytic cell line THP-1 and normal human PBMC were investigated.

Results from our *in vitro* binding studies suggested that SSCrFCES would be a potent inhibitor of the LPS activation of monocytes. To test this prediction, we measured the ability of SSCrFCES to inhibit hTNF-α and hIL-8 production by THP-1 cells incubated with 25 ng/ml and 100 ng/ml of LPS in a serum-free system containing various concentrations of SSCrFCES. THP-1 cells were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (0.1 mg/ml), at 37°C in a humidified environment in the presence of 5 % CO_2. The cells were maintained at a density between 2.5×10^5 and 2.5×10^6 cells/ml.

THP-1 cells were prepared for experiment by addition of a concentrated stock solution of phorbol myristate acetate (PMA, 0.3 mg/ml in dimethyl sulfoxide) to cell suspension to give a final concentration of 30 ng/ml PMA and 0.01% dimethyl sulfoxide (25). PMA-treated cell suspensions were immediately plated into 96-well microtitre plate at a density of 4×10^5 cells/ml and allowed to differentiate for 48 hours at 37°C. Immediately before stimulation by 25 ng/ml LPS or LPS pre-incubated with various concentrations of SSCrFCES, the culture medium was removed, and the cells were washed twice with serum-free RPMI 1640 and incubated at 37°C. At indicated times, the culture medium was collected. Human TNF-α and IL-8 concentrations in the supernatants were assayed using ELISA as suggested by the manufacturer.

Heparinised venous blood drawn from healthy donors was subjected to fractionation using Ficoll-Paque PLUS (Pharmacia) to obtain peripheral blood mononuclear cells (PBMC). PBMC were washed with PBS and suspended at a cell density of 1.5×10^6 cell/ml with RPMI 1640 medium supplemented with 10% FBS. PBMC were incubated at 37°C for 24 h at a density of 1.5×10^5 per well. LPS stimulation and immunoassay of hTNF-α and hIL-8 were performed as described for

THP-1 cells. In addition, the suppressive effect of SSCrFCES on LPS-induced cytokine release was investigated in the presence of 10% human serum. The difference between the test and control groups was subjected to Student's t-test. The values were obtained from at least three independent experiments.

Figure 5 shows that with THP-1 cells, 0.5 μM of SSCrFCES potently inhibited >90% LPS-induced production of TNF-α and IL-8 in the presence of high level of endotoxin. At 25 ng/ml LPS concentration tested, 0.7 μM of SSCrFCES is sufficient to completely prevent LPS-induced TNF-α production (Figure 5A). At 100ng/ml LPS, 1 μM of SSCrFCES reduced 90% IL-8 production as compared to control (Figure 5B).

Our findings indicate that 1 μM of SSCrFCES effectively prevent the LPS-mediated induction of hTNF-α and hIL-8 production by THP-1 when these cells were incubated in the presence of high endotoxin levels. It is important to note that the concentrations of LPS (25 ng/ml and 100 ng/ml) used in these studies are among the highest known concentrations reported for LPS-induced cytokine production. On molar basis, SSCrFCES appears to be more potent than polymyxin B and LF-33 at suppressing LPS-induced LAL coagulation and hTNF-α or hIL-8 secretion by THP-1 cells under serum-free conditions (24). This suggests that SSCrFCES has a much greater intrinsic capacity to neutralize endotoxin than polymyxin B. Again, it is attributable to its cooperative binding of LPS.

Purified human PBMC were used to test the suppression of endotoxin-induced TNF-α and IL-8 secretion by SSCrFCES under normal physiological conditions. In the absence of human serum, addition of only 0.1 μM of SSCrFCES completely inhibited TNF-α and IL-8 response to 10 ng/ml LPS by 50% (Figures 6A and 6B). When SSCrFCES was added to human serum (final concentration, 10%) before the addition of endotoxin, the suppressive effect of SSCrFCES was attenuated. It required 17 fold more SSCrFCES to suppress TNF-α and IL-8 secretion by 50%. A similar effect of human serum has also been observed with other cationic anti-endotoxin proteins such as LF-33 (24) and LALF (26). This is due to the interaction of these factors with serum proteins that effectively reduce their availability for binding to endotoxin. However, if the SSCrFCES was mixed with endotoxin 5 min before the addition of serum, the effect of the serum on the neutralization of endotoxin by SSCrFCES was

greatly reduced, requiring only 4 fold more SSCrFCES for 50% inhibition (Figures 6A and 6B).

Results from the *in vitro* binding studies suggested that the 4 Factor C-based sushi peptides would be potent inhibitors of the LPS-induced cytokine release by monocytes. To test this prediction, we measured the ability of S1, S1Δ, S3, and S3Δ to inhibit hTNF-α production by THP-1 cells incubated with 10 ng/ml of LPS in a serum-free system containing various concentrations of peptides.

As shown in Figure 6C, both modified peptides, S1Δ and S3Δ, are more potent inhibitors, giving 50% inhibition at 53.3 and 45.8 μM, respectively, as compared to the S1 and S3 peptides.

With the designed peptides (V1 and V2) 50% inhibition of LPS-induced TNF-α release were 27 and 35 μM, respectively.

Example 6: SSCrFCES and synthetic peptides are not cytotoxic to eukaryotic cells

In addition to high specific LPS binding, an important feature when using proteins for in vivo application to treat Gram-negative bacterial septic shock, are their physicochemical properties in biological systems. Problems that often arise in animal experiments are due to toxicity, as in the case of polymyxin B, or a very short half-life in the circulating system, for example BPI. To assess these features, we investigated SSCrFCES for their ability to permeabilize cultured cells.

Two x 10^4 THP-1 monocytes in 50 μl of RPMI 1640 were mixed in a microtitre plate with 50 μl of increasing amount of 2-fold serial dilutions of SSCrFCES (0.004 – 4.0 mg/ml in PBS) and incubated for 60 min at 37°C. To determine cytotoxicity induced by the SSCrFCES, 20 μl of CellTiter96™ AQ_{ueous} One Solution Reagent (Promega) was added into each well for 90 min at 37°C. [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) is bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium (27, 28). For detection, the absorbance was measured at 490 nm. To determine the ratio of cell lysis induced by SSCrFCES, two controls were used. Complete lysis (100%) was achieved by incubating cells in phosphate buffer saline containing 0.2 % Tween-20 instead of medium only. This

absorbance value corresponded to the background, as those cells could not metabolize MTS. The second control representing 0% lysis was determined by incubating cells in medium only. The LD_{50} was calculated as the concentration of SSCrFCES necessary to lyse 50% of the cells. The experiment was done in triplicate.

SSCrFCES had minimal effect on cell permeabilization (Figure 7). At the highest concentration of 4 mg/ml or 109 μM, only 20% cell lysis was observed. Compared to polymixin B where 50% cell lysis occurred with 0.51 mg/ml (29), this clearly indicates that SSCrFCES is a non-toxic anti-endotoxin protein. The synthetic peptides are non-cytotoxic.

Example 7: Pharmacokinetic analysis of SSCrFCES – Clearance rate in mice

600 μg of SSCrFCES was labeled with biotin using EZ-Link™ PEO-Maleimide Activated Biotin (Pierce) according to the manufacturer's instructions. The excess biotin was subsequently removed via ultrafiltration through Microcon-10 (Millipore). Three C57BL/6J mice were given a single i.v. bolus injection of 200 μg biotin-labeled SSCrFCES. Blood was collected from each of the 3 mice at time intervals over a 5-h period. The blood was immediately treated with SDS-PAGE loading dye and boiled for 5 minutes. The mixture was resolved in a 12% SDS-PAGE and electroblotted onto a PVDF membrane. Immunoblotting and hybridisation were carried out as described above except NeutrAvidin™-HRP antibody (Pierce) was used. Exposure time for chemiluminescence detection was extended to 1 hour. The signal on the X-ray film was quantitated via densitometric scan. The clearance rate of biotin-labeled SSCrFCES was analyzed using NCOMP, which is a WINDOWS-based program for noncompartmental analysis of pharmacokinetic data (30).

Densitometric scan revealed that significant amounts of circulating half-life of SSCrFCES is sufficiently long to allow easy detection during the first 90 minutes post-injection. NCOMP, which provides an interactive graphical environment for noncompartmental analysis of pharmacokinetics data by facilitating estimation of the zero and first moments of concentration-time data, was used for analysis. The calculated clearance rate of biotin-labeled SSCrFCES in C57BL/6J mice is 4.7 ml/min (Figure 8). The clearance rate is 2.7 fold slower than BPI. Therefore, a lower dose

of SSCrFCES would be adequate to maintain high enough circulating levels to compete with LBP for LPS.

Example 8: SSCrFCES and synthetic peptides neutralize LPS-induced lethality in mice

The anti-endotoxin potency of SSCrFCES was investigated in C57BL/6J mice. Mice are typically resistant to endotoxin. However, the sensitivity of mice to endotoxin can be enhanced > 1,000-fold by co-injection with a liver-specific inhibitor, galactosamine (31). In our study, intraperitoneal (i.p.) injection of 2.5 ng of *E. coli* 055:B5 LPS together with 15 mg of galactosamine hydrochloride in 0.2 ml of saline induced nearly 100% lethality in 18-25 g C57BL/6J mice within 7 hours. Various concentrations of SSCrFCES (1, 2, and 4 μM) and synthetic peptides (25 and 75 μg) were injected intravenously (i.v.) through tail vein 10 minutes after i.p. injection of the LPS-galactosamine mixture. Lethality was observed over 3 days after injection. Statistical analysis were performed using the Kaplan-Meier test (32) and log rank pairwise test.

As shown in Figure 9A, the LPS-induced lethality was reduced by 20% when 1 μM of SSCrFCES was injected i.v. 10 min after the i.p. injection of LPS. Higher concentrations of SSCrFCES of 2 and 4 μM conferred 90% and 100% protection, respectively.

A protective role of SSCrFCES viz LPS-binding domain of Factor C is thus shown in an intraperitoneal murine sepsis model. The mechanism by which SSCrFCES protects mice from LPS-induced sepsis is presumably mediated through its high affinity association to lipid A moiety of LPS, which consequently reduces the secretion of cytokines like TNF-α and IL-8. Figure 9B shows that S1, S1Δ, and S3 conferred 22-100% protection, whereas at 75 μg, S3Δ was most efficacious, giving 100% protection against LPS-induced toxicity.

Example 9: Antimicrobial action

Recently, the concept of eradication via targeted disruption of bacterial LPS by cationic peptides/proteins was introduced (33). For an effective antimicrobial therapy, such peptides need to satisfy several important criteria, including potent antimicrobial

activity over a wide range of pH, fast killing rate, low toxicity, and low hemolytic activity. While numerous antimicrobial peptides/proteins, like FALL-39 (34), SMAP-29 (35), lepidopteran cecropin (36), and CAP-18 (37) have been reported, few display all the above mentioned attributes. Thus, the search for new, more powerful and yet safe antimicrobial peptides continues to enrich the therapeutic armamentarium.

Further analysis of the sushi peptides showed them to have low cytotoxicity and to be capable of neutralizing LPS-biotoxicity (See Examples 4, 5, and 6 above) . This property provides a vital advantage over other antimicrobial peptides in suppressing adverse effects of LPS-induced septic shock during or after treatment.

Septic shock is characterized by a drastic fall in blood pressure, cardiovascular collapse, and multiple organ failure. Septic shock is responsible for over 100,000 deaths a year in the US alone. The septic shock condition (38, 39) often creates more complication than the actual infection itself when a massive amount of LPS is released by bacteria disintegrated by antibiotics. This problem is especially pronounced in children, in the elderly, and in immuno-compromised patients.

The present invention demonstrates novel and hitherto unsurpassed antimicrobial action of Factor C sushi peptides against clinical isolates of *P. aeruginosa*. Although the sushi peptides are demonstrated to be efficacious against this microorganism, antimicrobial potency is not limited to *P. aeruginosa* but should extend to any bacterium producing LPS bound by Factor C.

Antimicrobial action of SSCrFCES, SSCrFCsushi-GFP proteins and synthetic peptides (e.g., S1, S1Δ, S3, S3Δ, and V peptides), examined by microbiocidal concentrations (MBC_{90}) assays, show that these recombinant proteins and synthetic peptides have potent antimicrobial activities. Antimicrobial activity is expressly demonstrated against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and *Helicobacter pylori*. However, the antimicrobial acitivity of these proteins and peptides is not limited to only these three species of bacteria.

Peptides of 34 amino acids were synthesized based on the sequence of two regions of Factor C: sushi 1 and sushi 3, as well as their corresponding mutants (sushi 1Δ and sushi 3Δ), were found to harbour strong antimicrobial activities. Collectively, all four peptides (named S1, S1Δ, S3, and S3Δ) demonstrated exceptionally effective

bactericidal activity against gram-negative bacteria, represented by *Pseudomonas aeruginosa*.

At 0.03-0.25 μg/ml (8-63 nM), the MBC_{90} values of the peptides, are of the lowest ever reported against Pseudomonads. Viable bacteria were reduced by 90% after 7 minutes and were totally eradicated within 30-40 minutes. These peptides were minimally hemolytic against both rabbit and human erythrocytes (30%) at concentrations of 100 μg/ml (25 μM), which is up to 3333 times their effective MBC concentration.

These findings demonstrate the unprecedented therapeutic value of the sushi peptides and their mutants for treatment of *Pseudomonas* infections. Other sushi peptide derivatives (S4, S5) were also found to have variable antimicrobial activities. Thus, these results are given by way of example, and the present invention should not be deemed to be limited to only these representative peptides.

Test strains cultured on Mueller-Hinton agar (MHA, Becton Dickinson, USA) were inoculated into 10 ml Mueller-Hinton broth (MHB, Becton Dickinson, USA) and grown overnight at 37°C in a shaker incubator (Model 4536, Forma Scientific, Inc., USA) at 230 rpm. Overnight broth cultures were diluted to give a final cell density of 10^5 colony forming units/ml (cfu/ml). One hundred microliters of the bacterial suspension was dispensed into sterile polypropylene 8-strip PCR-tubes (Quality Scientific Plastics, USA). Eleven microliters of serially diluted sushi peptides, ranging in final concentrations of 0.03-4 μg/ml, were then added. The peptides were constituted at 10 times the required test concentrations in 0.01% acetic acid and 0.2% bovine serum albumin (BSA). Positive controls were cultures without test peptides. Uninoculated MHB was used as negative control. All tests were carried out in triplicate.

Cultures were incubated at 37°C for 18-24 h, with the PCR-tubes held in horizontal position and shaken at 230 rpm. Cell counts were determined by standard drop-count method. The killing efficiency for the four sushi peptides were calculated based on standard drop-count method. All four peptides (S1, SΔ1, S3, and SΔ3) showed potent bactericidal activity of <0.03-0.25 μg/ml against the 30 clinical strains of *P. aeruginosa* (Table 2).

The MBC_{50} determined for all the 4 peptides was <0.03 μg/ml (<7.5-8.0 nM). The MBC_{90} for the peptides were: <0.03 μg/ml (8 nM) for S1; 0.06 μg/ml (16 nM) for SΔ1; <0.03 μg/ml (8 nM) for S3; and 0.25 μg/ml (63 nM) for SΔ3. These MBC_{90} values are unsurpassed by any known antimicrobial peptides reported for *P. aeruginosa*. The MBC_{90} for the control strain of *P. aeruginosa* ATCC 27853 was 0.03 μg/ml (7.5-8.0 nM) for all the 4 peptides (Figure 12).

The antimicrobial therapeutic value of sushi peptides is exhibited by their exceptional bactericidal activity against gram-negative bacteria, e.g.: 30 clinical isolates and a control strain of *P. aeruginosa* ATCC 27853. The resistance pattern of these strains gave a close representation of the resistant strains of *P. aeruginosa* found in Singapore (Table 2).

The remarkably low MBC_{90} values of <0.03-0.25 μg/ml (<8.0-63 nM) obtained for the peptides are unsurpassed by any known antibiotics of metabolite or peptide origin. Comparatively, sushi peptides are 1-3 orders of magnitude more effective against *P. aeruginosa* than are other reported antimicrobial peptides. Owing to their high affinity for LPS, the sushi peptides probably exert anti-*Pseudomonas* effect through disruption of the LPS-lamellar organization.

Although, the peptides are targeted at the conserved lipid A domain, different MBCs were observed over the 30 clinical isolates. This is most likely due to differential permeability of the peptides into the variable polysaccharide components in the different *Pseudomonas* strains. This is supported by the different binding affinities of the sushi peptides for *Escherichia coli* B5:055 lipid A (See Example 3 of the present application).

The killing rate assay was adapted from the MBC test above, with different contact time of peptides with the bacteria arrested at regular intervals and plated for colony count. An initial density of 10^9 cfu/ml was used. Figure 13 shows that sushi peptides exhibit rapid bactericidal action. This is one of the important features of an effective therapeutic agent.

With an effectively low MBC_{90} concentration, we proceeded to investigate the killing time for the sushi peptides. At 0.06 μg/ml, all four peptides achieved MBC_{90} within 7 minutes. Within 30 minutes, the peptides totally eradicated an initial cell

population of 1 x 10^9 cfu/ml (Figures 13 and 14). *P. aeruginosa* is a fast-replicating bacteria, which displays a short lag phase and doubling time. Hence, a rapid bactericidal action is an extremely important factor especially with an infection that occur near or in vital organs like cornea (contact lens contamination in the eye), lung (in cystic fibrosis), and acute bacteraemia in AIDS patients. At a concentration of 0.06 ug/ml, the sushi peptides were able to eradicate 90% of viable cells within 7 min of incubation (Figure 13).

Complete eradication is assured to occur within the first two generations of bacteria which reduces the possibility of mutation. Thus, this rapid killing rate reduces the chance/opportunity for the development of resistance. Resistance will be remote as it will require several precise mutations occurring at multiple enzymes along the LPS synthesis pathway to ultimately yield a modified LPS structure that is sufficiently different to evade sushi peptide recognition. However, the possibility of developed or acquired resistance cannot be precluded if some of these strains are allowed to mutate at sub-lethal peptide concentrations.

Figure 15 shows electron micrographs illustrating how some multiple antibiotic-resistant strains of bacteria are killed by these peptides.

Human and rabbit erythrocytes were both used to test the hemolytic activities of the peptides. Whole blood was collected in heparinized sterile syringe, transferred to sterile borosilicate tube and centrifuged at 1200 g for 5 minutes at 4°C. The supernatant including the leukocytes above the erythrocyte pellet was discarded. The erythrocytes were washed 3 times using three volumes of pre-chilled pyrogen-free saline (PFS). An erythrocyte suspension at 0.4% was prepared for the hemolysis assay. Serial two-fold dilutions of the peptides was prepared in PFS and 100 μl aliquots were added to equal volumes of 0.4% erythrocyte solution in a 96-well microtiter plate (Nunclon™ Δ surface, Nunc) to give final peptide concentrations ranging from 6 to 100 μg/ml. The mixtures were incubated at 37°C for 1 h. The intact erythrocytes were then pelleted by centrifuging at 1000 g for 5 min. One hundred μl of the supernatant was transferred to a new 96-well microtiter plate and the amount of hemoglobin released into the supernatant was determined by reading the absorbance at 414nm using a SPECTRAmax™ 340 plate reader with SOFTmax

PRO™ version 1.2.0. A positive control with 100 μl of 0.4% erythrocyte lysed in 1% Triton-X 100 was taken as 100% lysis. The negative control was the erythrocytes in PFS alone, which gave minimal lysis. This was taken as 0%.

Figure 16 shows that sushi peptides have low hemolytic activity. This is crucial to the applicability of an antimicrobial agent for therapeutic use in humans and animals. Even at concentrations of 100 μg/ml (25 μM), up to 400-3333 fold of their MBC_{90}, the sushi peptides showed minimal hemolytic activity (Figure 16). On a separate assay, the hemolytic activity of sushi peptides was tested on rabbit erythrocytes. At the same concentration, the peptides showed hemolytic activity below 6%. For purposes of the present application, the language "substantially free of hemolytic activity" means showing hemolytic activity below 6%.

Thus, the ability of sushi peptides to: (a) cause effective LPS-neutralization (see Examples 4 and 5); (b) confer crucial protection against LPS-indcued lethanlity in mice (see Example 8); (c) possess low MBC_{90} values; (d) induce rapid killing rate; and (e) exhibit lack of hemolytic activity, are features that indicate that these peptides will provide great advantages over currently available antibiotics.

With this invention, the LPS toxicity during the course of treatment will be dramatically reduced. The sushi peptides will provide highly effective and potentially useful therapeutics for the treatment of *P. aeruginosa* infections. It leaves very little doubt that these peptides will be equally effective against other members of Pseudomonads.

Example 10: SSCrFCsushi-GFP proteins bind LPS and gram-negative bacteria

The recombinant SSCrFCESsushi-GFP proteins were able to bind/tag gram-negative bacteria, showing as green fluorescent tagged organisms. This makes a convenient detection tag for displaying such microogranisms in samples.

Example 11: LPS-affinity chromatography (for removal of endotoxin from liquid samples)

By way of an example, S3Δ peptide (with Kd of $10^{-7 \text{ to } -8}$ M) was chosen from amongst the sushi peptides to create an affinity chromatography system to display

the power of binding of LPS from liquid samples. Thus, a solution of 4 mg/ml of S3Δ (in conjugation buffer: 0.1 M MES [2-(N-Morpholino)ethanesulfonic acid], 0.9% NaCl, pH 4.7) was immobilized via EDC [1-ethyl-3-(3-dimethylaminopropyl) cardiimide] / DADPA (Diaminodipropylamine), obtained from Pierce Chemicals, USA). After 3 hours of conjugation to DADPA-Agarose CL-6B in a small column, the flowthrough was collected and the absorption of fractions at 280 nm was measured to calculate the total amount of peptide immobilized to the matrix (by substraction from the unbound S3Δ found in the flowthrough).

It was found that binding efficiency of S3Δ to the EDC-activated resin was 50%. After regeneration of the column with 5 column volumes of 1% sodium deocycholate (DOC) - to ensure the removal of any exogenous LPS that may be bound to the resin, and washing the resin with pyrogen-free water, the column was ready for LPS absorption.

Again, by way of example, two 50 ml volumes of LPS solution (either LPS from Sigma, or FITC-labelled LPS from List Biologicals) containing 1 and 0.05 EU/ml were loaded onto the column. In each case, the flowthrough was subjected to LPS measurement by either LAL kinetic-QCL kit (BioWhittaker) or spectrofluorimetry, depending of the type of LPS solution that was used. In each case, the level of unbound LPS remaining in the flowthrough was below the detection limit (0.005 EU/ml) of the LAL kinetic-QCL assay. The affinity column was re-usable repeatedly, using 1% DOC as a regenerating agent.

LPS-affinity chromatography was also demonstrated by batchwise chromatography using 0.5 ml of 0.5 ug/ml FITC-LPS solution (in different buffers). The resin suspension was rotated for 3 h at room temperature, briefly spun at 1000 rpm for 1 min and the supernatant was reclarified at 12000 rpm for 10 minutes. The resultant supernatant was measured for unbound FITC-LPS by spectrofluorimetry. Figure 17 shows S3Δ peptide-FITC-LPS coupled agarose beads seen under UV-fluorescence microscope.

The optimal binding of LPS to S3Δ was tested under different pH conditions and ionic strengths. Binding decreases with increase in ionic strength, and increases with increase in pH (Figure 18). Thus, the best condition for binding of LPS to the

affinity resin is basic and low ionic strength conditions. The optimal condition is expected to vary with different protein solutions.

Purified SSCrFCsushi-GFP proteins can also be chemically-linked to activated resins via their C-terminus GFP region, to allow N-terminal LPS-binding domain to be exposed for capturing endotoxin when an LPS-contaminated solution or biological fluid is passed through the resin.

Example 12: Production of rFC in *P. pastoris*

In this study, the cloned Factor C cDNA of the Singapore horseshoe crab, *Carcinoscorpius rotundicauda* (10), was expressed in a methylotrophic yeast, *Pichia pastoris*. The full-length rFC so produced was found to lack serine protease activity, yet possess a functional endotoxin-binding domain. The full-length rFC from *P. pastoris* is able to bind free or bound LPS. Deletion proteins rFCEE and rFCSN containing the 5′ and 3′ regions, respectively, of Factor C were also produced and assayed for lipid A binding activity. The presence of a fully functional endotoxin-binding domain on the full-length rFC, and a slightly reduced endotoxin-binding capacity in rFCEE was demonstrated by two modified qualitative and quantitative LPS binding assays.

A. Materials and Methods

(1) Glassware and Buffers

All glassware was rendered pyrogen-free by baking at 200 °C for 4 h. Buffers were prepared using pyrogen-free water (Baxter) and autoclaved at 121°C for 2 h. Sterile disposable plasticware was used whenever possible. Other non-heat resistant apparatus was soaked in 3% hydrogen peroxide before rinsing with pyrogen-free water and drying in an oven.

(2) Recombinant Factor C Constructs

Three recombinant Factor C constructs -- pHILD2/CrFC21, pHILD2/CrFC21EE, and pPIC9/CrFC26SN (10,12,75) -- were used for the study. As a control, pHILD2/151, an isolate of *P. pastoris* containing only the parent

vector, pHILD2, was also included. pHILD2/CrFC21 contains the full-length CrFC21 cDNA (GenBank Database Accession No. S77063) of 3.4 kb together with its native translational start and signal sequence while pHILD2/CrFC21EE contains the 2.3 kb 5' EcoRI fragment isolated from CrFC21 cDNA. This construct contains the 762 amino-acid fragment encompassing the heavy chain of CrFC21 along with its endotoxin-binding domain. The pPIC9/CrFC26SN construct contains the 2.4 kb 3' SalI-NotI fragment of CrFC26 (GenBank Database Accession No. S77064) cloned as a fusion fragment, in-frame and downstream of the pPIC9 vector start site and secretion signal. CrFC26SN contains sequence similar to the corresponding fragment in CrFC21 (10). This is a truncated construct lacking the putative LPS-binding domain and therefore, serves as a useful negative control in LPS-binding assays. The recombinant Factor C proteins from pHILD2/CrFC21, pHILD2/CrFC21EE, and pPIC9/CrFC21SN are referred to as rFC, rFCEE, and rFCSN, respectively.

(3) Growth Conditions

Recombinant *Pichia* clones of pHILD2/CrFC21, pHILD2/CrFC21EE and pPIC9/CrFC26SN as well as the negative control, pHILD2/151 were grown overnight in shake flasks at 300 rpm and 30°C in 1 L MGY growth medium containing 1.34% yeast nitrogen base (Difco), 1% glycerol and 4 x 10^{-5}% biotin. At the mid-log phase of growth (OD_{600} 2.0), the yeast cells were harvested aseptically at 3,000 x g for 10 min and transferred to 2 L MM induction medium, containing 1.34% yeast nitrogen base (Difco), 0.5% methanol and 4 x 10^{-5}% biotin. Induction was carried out at 30°C for 8 h. Induced cells were harvested by centrifugation at 3,000 x g for 10 min.

(4) Preparation of rFC samples from recombinant yeast clones

Induced yeast cells were disrupted by 10 cycles of nebulization (Glas-Col™ BioNeb) at 200 psi using purified N_2. Soluble and insoluble fractions were separated by centrifugation at 13,200 x g for 12 min. The supernatant containing soluble proteins was partially purified by ammonium sulfate precipitation at 20%

saturation and resuspended in 50 mM Tris-Cl buffer, pH 8. The mixture was desalted through a Sephadex™ G-25 column (Pharmacia) equilibrated in the same buffer. In a separate preparation, the crude yeast supernatant was subjected to ultrafiltration through a Biomax™-50 (Millipore) membrane. The Biomax™-50 enriched rFC was further purified by chromatography through a Sephadex™ G-100 column (1x25 cm; Pharmacia). Total protein was measured by Bradford assay (62).

(5) Western Analysis of rFC Protein

rFC samples were electrophoresed on denaturing 10% SDS-polyacrylamide gel (63) and electroblotted onto Immobilon™ PVDF membrane. The respective rFC was immunolocalized by incubating the blot with rabbit anti-Factor C primary antibody and visualizing with horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (Dako) using 4-chloro-1-naphthol and H_2O_2 as substrate.

(6) LPS-Binding Assay of rFC

LPS from *E. coli* 055:B5 (Sigma) was reconstituted to 2 μg/μl, and diphosphoryl lipid A from *E. coli* K12, D31m4 LPS (List Biologicals, Inc., USA) was made up to 1 μg/μl. The LPS-binding assay was based on modifications of earlier described protocols (45,61). Briefly, 10 μg aliquots of LPS/lipid A were electrophoresed on a denaturing 15% SDS-polyacrylamide gel and electroblotted onto Immobilon™ PVDF membrane. The membrane was cut into strips and each LPS/lipid A strip was subsequently incubated with 300 μg of proteins containing rFC. Detection of rFC binding to lipid A was accomplished by incubation with anti-Factor C antibody followed by alkaline phosphatase-conjugated secondary goat anti-rabbit antibody (Dako) and BCIP/NBT colorimetric substrate (Moss, Inc., USA).

(7) Assay for competition between rFC and CAL Factor C for LPS

Carcinoscorpius amoebocyte lysate (CAL) containing native Factor C was used in an assay in which rFC competed with CAL Factor C for LPS. Because the

rFC produced in *P. pastoris* lacks serine protease activity, the competition can be monitored by measuring the reduced enzymatic activity of CAL in a fluorimetric assay. Mixtures of 100 μl each of increasing concentrations of LPS or rFC, in fluorimetric assay buffer (50 mM Tris HCl pH 8, containing 0.1 M NaCl and 0.01 M $CaCl_2$) were incubated at 37°C for 1 h. Aliquots of 20 μg CAL were added to each mixture and the total volumes were made up to 2 ml with fluorimetric assay buffer. The reaction was continued at 37°C for 1 h and the fluorimetric assay protocol (64) was followed. This involved the addition of 15 μl of 2 mM fluorimetric substrate N-t-Boc-Val-Pro-Arg-7-amido-4-methylcoumarin (Sigma) and incubation at 37°C for 30 min. The reaction was terminated by the addition of 0.1 ml of glacial acetic acid (Merck). The product, amino methylcoumarin, was measured in fluorescence units (FU) on a Luminescence Spectrometer LS-5 (Perkin-Elmer) with excitation light at 380 nm and emission at 460 nm.

(8) Binding Interactions Between rFC and its Immobilized Ligand, Lipid A

The binding interactions between rFC and immobilized lipid A were monitored using the BIACORE X™ biosensor (Pharmacia Biotech). The BIACORE X™ sensor chip features a flat hydrophobic surface that allows the immobilization of ligand molecules. Thirty microliters of lipid A at 100 μg/ml were immobilized on each sensor chip to form a ligand surface. Biomax™-50 enriched samples of rFC, rFCEE and rFCSN, each at 1 mg/ml were injected at 10 μl/min for 3 min over the ligand surface. After each injection of the recombinant protein samples, the lipid A ligand surface was regenerated using 0.1 M NaOH. The ligand-binding was measured in relative response units (RU) for each sample, and calculated from the difference in RU at the baseline, viz., before injection of sample, and final experimental reading taken after sample injection and a 2-min wash. The percentage binding was thus determined.

B. Results and Discussion

Nebulization of *P. pastoris* clones released soluble and bioactive rFC.

After nebulization, the supernatant derived from clarification at 13,200 x g of the *P. pastoris* cell lysate contained soluble forms of rFC and rFCSN of 132 and 89 kDa protein bands, respectively (Fig. 19). Compared to glass bead treatment (61), nebulization enhanced the breakage efficiency of *P. pastoris*. Furthermore, the rFC was fractionated into the soluble phase, thus enabling its direct use for functional analysis, as well as ease of purification. This is a significant improvement over the earlier rFC preparations from glass-bead breakage where insoluble rFC had to be solubilized by treatment with detergents (12). Detergent-solubilization, in particular, with Triton X-100 has been reported to inhibit Factor C binding of LPS (68). We have also shown that SDS at > 0.5 % also inhibits the activity of Factor C in CAL. Removal of SDS using potassium chloride (69) restores the LPS binding activity of solubilized rFC. However, care must be taken to avoid pyrogenation. Thus, it is best to obtain soluble rFC under pyrogen-free conditions via physical methods and not chemical means.

Using either $(NH_4)_2SO_4$ precipitation or Biomax™-50 ultrafiltration, the rFC preparation was enriched in total protein content. Chromatography of Biomax™-50 rFC through a Sephadex™ G-100 molecular sieve further purified rFC from other yeast proteins (Fig. 20).

In the modified Western blot of LPS, rFC was shown to bind to the lipid A moiety displaying specific bands in the range of 7 - 20 kDa (Fig. 21A) which is consistent with previous findings (43,70). Subsequently, when the modified Western blot of diphosphoryl lipid A was used, the specificity of rFC for lipid A was further confirmed (Fig. 21B). Recombinant Factor C samples derived from $(NH_4)_2SO_4$ precipitation; Biomax™-50 ultrafiltration and Sephadex™ G-100 gel filtration displayed increasing affinity for lipid A (Figs. 21A and 21B : Lanes 3 & 4). No binding to the 7 - 20 kDa bands was observed with rFCSN and pHILD2/151. Biomax™-50 enriched rFCEE, the truncated Factor C, was also able to bind specifically to lipid A moiety of LPS, albeit less strongly (Figs. 21A and 21B).

The presence of a functional LPS-binding domain demonstrates that rFC expressed in yeast folds properly, or at least its endotoxin-binding domain does so. The postulated endotoxin-binding region of the *C. rotundicauda* Factor C (61)

is located in the amino terminus of the heavy chain, which comprises the cysteine-rich EGF-like domain and one or two sushi domains (16,71). Recombinant Factor C specifically binds to the lipid A moiety showing the ability of rFC to recognize and bind the biologically-potent moiety of LPS. That the binding of LPS to Factor C requires the presence of an endotoxin-binding domain was confirmed using the rFCSN where the lack of 5'-terminal LPS-binding domain in this deletion homolog resulted in its inability to bind lipid A. The observable reduction in intensity of binding of rFCEE to lipid A as compared to that of rFC indicates that although the binding of lipid A requires the presence of the LPS-binding domain, sequences of Factor C further downstream may mediate the strength of the binding. It has been reported that binding of endotoxin triggers a conformational change in the Factor C molecule (58) where downstream sushi domains are involved in protein-protein interaction.

Recombinant Factor C competes for LPS, causing reduction in enzymatic activity of CAL Factor C. The yeast rFC, lacking serine protease activity, but capable of binding to LPS, was observed to compete with CAL native Factor C for the LPS. This resulted in the depletion of LPS available to bind native Factor C, thus causing a reduction in its enzymatic activity (55).

The percentage competition by rFC of the native Factor C enzyme activity of CAL was calculated based on comparison of enzyme activity with the negative control of pHILD2/151. The following formula was employed:

$$\frac{(\text{FU of LPS in pHILD2/151+CAL})-(\text{FU of LPS in rFC+CAL}) \times 100\%}{\text{FU of LPS in pHILD2/151+CAL}}$$

where FU represents fluorescence units.

The competitive effect of crude rFC on LPS-activated CAL Factor C enzymatic activity was compared with the two partially purified rFC samples based on the above formula. Partial purification of rFC using $(NH_4)_2SO_4$ precipitation improved its competitive effect from 30% to 60%. Enrichment of rFC through Biomax™-50 improved its inhibitory efficiency to 81%.

A checkerboard analysis of fixed amount of rFC with variable concentrations of LPS was used to investigate binding efficacy and the ratio of interaction between rFC and LPS molecules. A comparison was made between the binding efficacy of crude rFC and Biomax™-50 rFC to LPS. Figure 4A shows that increasing amounts of rFC resulted in greater depletion of LPS, leading to an increase in the percentage loss of CAL Factor C activity. On the other hand, regardless of any fixed amount of rFC in the reaction mixture, increasing levels of LPS increased CAL Factor C activity. This indicates that excess LPS was again able to activate CAL Factor C enzyme activity. Even without purification, the crude rFC was able to effectively reduce Factor C activity in CAL by >80%, equivalent to a ratio of 1000 : 1 molecules of rFC to LPS (Fig. 22A, dashed line).

With increasing amounts of LPS used over a fixed amount of Biomax™-50 enriched rFC sample, the percentage loss of CAL Factor C activity was effectively maintained at >80% (Fig 22B). For a >80% reduction in CAL Factor C activity, a ten-fold increase in the binding efficacy was observed between enriched rFC and LPS at a ratio of 100 : 1 (Fig. 22B, dashed line).

The Biomax™-50 rFCSN which served as the internal negative control showed little or no effect on the enzymatic activity of CAL Factor C. The rFCEE, having a lower binding affinity of LPS, displayed a markedly lower competitive effect on the enzymatic activity of native Factor C (Fig. 23).

Only micrograms of the total crude rFC were needed to remove nanograms of LPS, as reflected by ≥80% loss in CAL activity. With partial purification and concentration using Biomax™-50 membrane, the ratio of rFC to LPS for maximal LPS removal improved by ten-fold.

Interactive binding between rFC and immobilized lipid A using the BIACORE X™ sensor indicates that although background binding was attributed to rFCSN, the overall binding of partially-purified rFC to lipid A gave a net response of approximately 30% of the total immobilized lipid A. Thus, the full-length rFC has an affinity for lipid A such that 30% of lipid A is bound by the partially-purified rFC when the ratio of partially-pure rFC to lipid A is 10:1 on a mass basis. This shows that rFC has affinity for bound lipid A. rFCEE also gave a binding response but

again, displayed at a lower affinity of 15% (Fig. 24). For purposes of this application, the terminology "retains lipid A binding activity" indicates an affinity of 10^{-6} M or lower. Preferably peptides will have a binding affinity of 10^{-7} M or lower.

In experiments described in reference 77, rFCES produced in Drosophila cells and purified to homogeneity shows 92% saturation of immobilized lipid A in a BIACORE X™ apparatus under conditions similar to those decribed above, except that the concentration of rFCES was 8 ng/µl. Thus, under these conditions 240 ng of rFCES binds 2.8 µg lipid A giving a ratio of about 1:12 rFCES to lipid A on a mass basis.

<u>Example 13: rFC has Bacteriostatic Activity</u>

The rFC and deletion proteins expressed in *P. pastoris* in Example 12 were examined for bacteriostatic activity in in vitro cultures.

The bacteria used for the assay were *Escherichia coli* ATCC#25922, Salmonella typhimurium ATCC#14028, *Pseudomonas aeruginosa* ATCC#27853, *Klebsiella pneumoniae* ATCC#13883 and *Staphylococcus aureus* ATCC#25923. A colony of each Gram-negative bacterium was inoculated into nutrient broth (Gibco, BRL) and grown at 37°C until it reached the logarithmic phase of growth. The culture was diluted with nutrient broth to give 1 - 5 x 10^5 cells/ml. Aliquots of 2 ml culture were incubated with 1 mg rFC/ml of culture. Incubation was carried out at 37°C At time intervals of 0, 2, 4, 6, and 24 h, the bacterial culture was vortexed to break up any agglutinated clumps. After vortexing, each culture was examined under the microscope to ensure homogeneity of bacteria. The culture was serially diluted with 0.85% saline, plated on nutrient agar (Oxoid) and incubated overnight at 37°C for colony counting.

Crude full-length rFC and Sephadex™ G-100 enriched rFC truncates rFCEE and rFCSN did not have any inhibitory effect on the growth of the various bacteria used. However, enrichment of full-length rFC by Biomax™-50 ultrafiltration followed by chromatography through Sephadex™ G-100 yielded rFC which inhibited the growth of Gram-negative bacteria such as *E. coli, K. pneumoniae, P.*

aeruginosa, and *S. typhimurium* (Fig. 25). The enriched rFC sample showed a particularly potent bacteriostatic effect on *K. pneumoniae*. This appears consistent with the antibacterial activity found in the cell-free hemolymph of *C. rotundicauda* (72). Further purification of rFC is expected to improve its bactericidal potential. The bacteriostatic effect was maintained at 100% for 2 h but the effect started to decline at 6 h of incubation, and was completely lost after 24 h. *S. aureus*, a Gram-positive bacterium, was not inhibited at all by rFC.

Agglutination of the bacteria was observed within 2 h of incubation with rFC. Figs. 26A-26E show a typical agglutination reaction exemplified by *E. coli*. This could be attributed to the bacteriostasis of the bacteria because interestingly, there was no agglutination observed with the *S. aureus* culture which similarly did not show any inhibited growth. Indirectly, the agglutination effect of rFC could be utilized as a rapid detection method and/or for the removal of Gram-negative bacteria from a sample.

Since LPS is required for reproduction of Gram-negative bacteria (40), it could be envisaged that rFC binds to the lipid A portion of LPS to neutralize its biological activities, causing agglutination which leads to bacteriostasis. This specific binding of rFC to LPS was confirmed by the observation that growth of Gram-positive *S. aureus*, which does not possess LPS on its outer wall, was not affected by rFC.

<u>Example 14: rFC Protects Actinomycin D-sensitized Mice from LPS Lethality</u>

Actinomycin D (Sigma) was used to sensitize mice to submicrogram amounts of LPS (65,66). The protective effect of rFC on the mortality of actinomycin D-sensitized/LPS-challenged mice was studied according to protocols previously described (19,67,21). In this *in vivo* experiment, 500 μl aliquots containing 25 or 50 ng LPS from *E. coli* 055:B5 (Sigma) and 50 μg rFC produced as in Example 12, or saline, were preincubated at 37°C for 60 min. The LPS-rFC or LPS-saline mix was combined 1:1 with 250 μg/ml actinomycin D immediately prior to injection. A 0.2 ml volume of this solution containing 25 μg actinomycin D, 5 or 10 ng LPS and 10μg rFC was injected intraperitoneally into each outbred male

Swiss albino (20-25 g) mouse. Groups of 10 mice were used for each replicate set of experiment. The percentage of surviving mice was determined at 72 h.

An earlier experiment determined that the 50% lethal dose of LPS on Swiss albino mice is 3.16 ng. Amounts of 5 and 10 ng of LPS were therefore used in this in vivo experiment. The protective effect of Sephadex™ G-100 enriched rFC on actinomycin D-sensitized/LPS-challenged mice is shown in Fig. 27. Recombinant Factor C was able to attenuate the toxic effect of LPS, and this resulted in the decreased mortality of the sensitized mice challenged with the rFC-LPS mix. On the other hand, rFCSN that lacks the endotoxin-binding domain did not confer any protection on the LPS-challenged mice. This observation suggests that like LALF (19,67) and human cationic antimicrobial protein CAP18 (21), rFC binds specifically to the biological moiety of LPS to neutralize its lethal effect on mice.

Example 15: LPS Binding by rFC Produced in a Baculovirus Host-vector System

Recombinant Factor C was produced in Sf9 cells infected with a recombinant baculovirus comprising the CrFC21 cDNA encoding full-length rFC. The recombinant protein was expressed and partially purified by Biomax™ ultrafiltration and gel filtration chromatography over Sephadex™ G-100 as described in reference 76. The recombinant rFC exhibits its normal serine protease activity as shown in that reference.

The partially purified, full-length rFC was assayed for activity in binding the LPS obtained from *K. pneumoniae*, *S. minnesota*, *E. coli*, and *S. typhimurium* using the BIACORE X™ system and the experimental conditions indicated in Example 12(8). The data of Fig. 29 show that the rFC binds much more strongly to the LPS from *K. pneumoniae* than to the LPS from *E. coli*.

Example 16: rFC Produced in Baculovirus-infected Cells Induces Bacteriostasis and Protects Mice from LPS Lethality

The rFC produced in the baculovirus-infected Sf9 cells described in Example 15 was assayed for its activity in inducing bacteriostasis and for protective effect in the LPS challenge experiment.

The bacteriostatic activity of the rFC from Sf9 cells was assessed in the manner described in Example 13. The data in Fig. 30 show that the bacteriostatic effect is observed and that, for *E. coli* and *K. pneumoniae*, it shows similar kinetics to that shown for the rFC obtained from yeast. The bacteriostasis induced for *S. typhimurium* and *P. aeruginosa*, on the other hand, was of much shorter duration when rFC produced in the Sf9 cells is used.

The ability of the rFC produced in recombinant baculovirus-infected Sf9 cells to protect mice from lethal LPS challenge was also tested. The experiment was conducted in the same manner as described in Example 14, except that only 10 μg of the partially purified rFC was administered. In Fig. 31, rFC-Sf9 indicates administration of recombinant rFC together with the indicated amount of LPS; wt-Sf9 indicates that supernatants from Sf9 cells harboring only wild type baculorvirus were used. Consistent with the bacteriostatic effect observed in cultured bacteria, the rFC produced in recombinant baculovirus-infected Sf9 cells was able to protect a significant proportion of the challenged mice from LPS lethality.

The invention being thus described, various modifications of the materials and methods used in the practice of the invention will be readily apparent to one of ordinary skill in the art. Such modifications are considered to be encompassed by the scope of the invention as described in the claims below.

Table 1. A comparison between binding affinity of Factor C-derived Sushi proteins and other LPS-binding proteins to lipid A

Proteins	Ligand	Association constant ($M^{-1}s^{-1}$)	Dissociation constant (s^{-1})	Equilibrium constant (M)	References
Sushi-123	**Lipid A *E.coli* K12**	**4.01×10^5 5.20×10^5**	**1.48×10^{-4} 7.88×10^{-7}**	**3.691×10^{-10} 1.515×10^{-12}**	
Sushi-1	**Lipid A *E.coli* K12**	**2.401×10^4**	**$3.64\ 10^{-6}$**	**1.516×10^{-10}**	
Sushi-3	**Lipid A *E.coli* K12**	**1.479×10^5**	**2.031×10^{-4}**	**1.373×10^{-9}**	
Native LALF	Lipid A *E.coli* K12	3.124×10^4	1.154×10^{-4}	3.694×10^{-9}	
Cationic protein 18 (CAP18)	LPS *S. minnesota* Re595			5.8×10^{-10}	de Haas *et al.*, 1998
Bacterial/Permeability-Increasing Protein (BPI)	Lipid A *E. coli* J5			4.1×10^{-9}	Gazzano-Santoro *et al.*, 1992
Recombinant BPI_{23}	Lipid A *E. coli* J5			$2.6\text{-}4.3 \times 10^{-9}$	Gazzano-Santoro *et al.*, 1992; 1994
Recombinant BPI_{21} (rBPI21)	LPS *S. minnesota* Re595			3.75×10^{-9}	de Haas *et al.*, 1998
BPI pep85-99 (15 mer)	"			1.76×10^{-6}	de Haas *et al.*, 1998
Serum amyloid P component (SAP)	LPS *S. minnesota* Re595			3.9×10^{-9}	de Haas *et al.*, 1998
SAP pep186-200	LPS *S. minnesota* Re595			1×10^{-5}	de Haas *et al.*, 1998
Native LBP	LPS *S. minnesota* Re595			3.5×10^{-9}	Tobias et al., 1995
Recombinant LPS-binding protein (LBP)	Lipid A *E. coli* J5			5.8×10^{-8}	Gazzano-Santoro *et al.*, 1994
NH-LBP (aa 1-197)				$\leq 1 \times 10^{-8}$	Han *et al.*, 1994
Recombinant soluble human CD14	LPS *S. minnesota* Re595			2.9×10^{-8}	Tobias *et al.*, 1995
Polymixin B				3.3×10^{-7}	Vaara M., 1992
Polymixin nonapeptides				$1.1\text{-}1.3 \times 10^{-6}$	Vaara and Vijanen, 1985
Limulus endotoxin-binding protein-protease inhibitor	LPS *E. coli* 055:B5			6×10^{-6}	Minett et al., 1991

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Asp Tyr Val Gln Val Arg Glu Ala Leu Glu Ile His Val Asn Pro Asn
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tac gac ccc ggc aat ctc aac ttt gac ata gcc cta att caa ctg aaa 3376
Tyr Asp Pro Gly Asn Leu Asn Phe Asp Ile Ala Leu Ile Gln Leu Lys
925 930 935

act cct gtt act ttg aca aca cga gtc caa cca atc tgt ctg cct act 3424
Thr Pro Val Thr Leu Thr Thr Arg Val Gln Pro Ile Cys Leu Pro Thr
940 945 950

gac atc aca aca aga gaa cac ttg aag gag gga aca tta gca gtg gtg 3472
Asp Ile Thr Thr Arg Glu His Leu Lys Glu Gly Thr Leu Ala Val Val
955 960 965

aca ggt tgg ggt ttg aat gaa aac aac acc tat tca gag acg att caa 3520
Thr Gly Trp Gly Leu Asn Glu Asn Asn Thr Tyr Ser Glu Thr Ile Gln
970 975 980

caa gct gtg cta cct gtt gtt gca gcc agc acc tgt gaa gag ggg tac 3568
Gln Ala Val Leu Pro Val Val Ala Ala Ser Thr Cys Glu Glu Gly Tyr
985 990 995 1000

aag gaa gca gac tta cca ctg aca gta aca gag aac atg ttc tgt gca 3616
Lys Glu Ala Asp Leu Pro Leu Thr Val Thr Glu Asn Met Phe Cys Ala
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ggt tac aag aag gga cgt tat gat gcc tgc agt ggg gac agt gga gga 3664
Gly Tyr Lys Lys Gly Arg Tyr Asp Ala Cys Ser Gly Asp Ser Gly Gly
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cct tta gtg ttt gct gat gat tcc cgt acc gaa agg cgg tgg gtc ttg 3712
Pro Leu Val Phe Ala Asp Asp Ser Arg Thr Glu Arg Arg Trp Val Leu
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gaa ggg att gtc agc tgg ggc agt ccc agt gga tgt ggc aag gcg aac 3760
Glu Gly Ile Val Ser Trp Gly Ser Pro Ser Gly Cys Gly Lys Ala Asn
1050 1055 1060

cag tac ggg ggc ttc act aaa gtt aac gtt ttc ctg tca tgg att agg 3808
Gln Tyr Gly Gly Phe Thr Lys Val Asn Val Phe Leu Ser Trp Ile Arg
1065 1070 1075 1080

cag ttc att tgaaactgat ctaaatattt taagcatggt tataaacgtc 3857
Gln Phe Ile

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Val Phe Tyr Ser Phe Pro Phe Val Lys Ser Thr Val Val Leu Leu Gln
35 40 45

Cys Tyr His Tyr Ser Leu His Asn Thr Ser Lys Phe Tyr Ser Val Asn
50 55 60

Pro Asp Lys Pro Glu Tyr Ile Leu Ser Gly Leu Val Leu Gly Leu Leu
65 70 75 80

Ala Gln Lys Met Arg Pro Val Gln Ser Lys Gly Val Asp Leu Gly Leu
85 90 95

Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
100 105 110

Phe Asn Ile Pro Val Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
115 120 125

Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys
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Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu Asp Ser Cys Val Thr
145 150 155 160

Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
165 170 175

Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Ala Cys Arg
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Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu Lys Gly Cys Pro Leu
195 200 205

Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn
210 215 220

Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
225 230 235 240

Met Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Asn Phe Pro

245 250 255

Pro Lys Cys Ile Arg Glu Cys Ala Met Val Ser Ser Pro Glu His Gly
260 265 270

Lys Val Asn Ala Leu Ser Gly Asp Met Ile Glu Gly Ala Thr Leu Arg
275 280 285

Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
290 295 300

Cys Gln Gly Asn Gly Gln Trp Asn Gly Gln Ile Pro Gln Cys Lys Asn
305 310 315 320

Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Lys
325 330 335

Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
340 345 350

Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asp
355 360 365

Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser
370 375 380

Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val
385 390 395 400

Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
405 410 415

Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
420 425 430

Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Leu Pro
435 440 445

Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp
450 455 460

Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser Glu Glu Leu Lys Ser
465 470 475 480

Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Ser Ser Ser Thr Ala Gly
485 490 495

Lys Ser Gly Cys Pro Asp Gly Trp Phe Glu Val Asp Glu Asn Cys Val

500 505 510

Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val Cys
515 520 525

Thr Asn Met Ala Ala Arg Leu Ala Val Leu Asp Lys Asp Val Ile Pro
530 535 540

Asn Ser Leu Thr Glu Thr Leu Arg Gly Lys Gly Leu Thr Thr Thr Trp
545 550 555 560

Ile Gly Leu His Arg Leu Asp Ala Glu Lys Pro Phe Ile Trp Glu Leu
565 570 575

Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn Leu Thr Phe Trp Ala
580 585 590

Ser Gly Glu Pro Gly Asn Glu Thr Asn Cys Val Tyr Met Asp Ile Gln
595 600 605

Asp Gln Leu Gln Ser Val Trp Lys Thr Lys Ser Cys Phe Gln Pro Ser
610 615 620

Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg Asn Lys Ala Lys Cys
625 630 635 640

Asp Asp Pro Gly Ser Leu Glu Asn Gly His Ala Thr Leu His Gly Gln
645 650 655

Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile Arg Tyr Ser Cys Glu
660 665 670

Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val Thr Cys Thr Thr Asn
675 680 685

Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile Lys Val Ile Thr Cys
690 695 700

Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val Glu Ile Lys Pro Pro
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Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser Pro Phe Leu Arg Leu
725 730 735

Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala Lys Pro Pro Pro Lys
740 745 750

Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu Ala Ser Lys Val Lys

gat agt tgt gtt act tgt cca cct aac aaa tat ggt act tgg tgt agc 338
Asp Ser Cys Val Thr Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser
95 100 105

ggt gaa tgt cag tgt aag aat gga ggt atc tgt gac cag agg aca gga 386
Gly Glu Cys Gln Cys Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly
110 115 120

gct tgt gca tgt cgt gac aga tat gaa ggg gtg cac tgt gaa att ctc 434
Ala Cys Ala Cys Arg Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu
125 130 135

aaa ggt tgt cct ctt ctt cca tcg gat tct cag gtt cag gaa gtc aga 482
Lys Gly Cys Pro Leu Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg
140 145 150 155

aat cca cca gat aat ccc caa act att gac tac agc tgt tca cca ggg 530
Asn Pro Pro Asp Asn Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly
160 165 170

ttc aag ctt aag ggt atg gca cga att agc tgt ctc cca aat gga cag 578
Phe Lys Leu Lys Gly Met Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln
175 180 185

tgg agt aac ttt cca ccc aaa tgt att cga gaa tgt gcc atg gtt tca 626
Trp Ser Asn Phe Pro Pro Lys Cys Ile Arg Glu Cys Ala Met Val Ser
190 195 200

tct cca gaa cat ggg aaa gtg aat gct ctt agt ggt gat atg ata gaa 674
Ser Pro Glu His Gly Lys Val Asn Ala Leu Ser Gly Asp Met Ile Glu
205 210 215

ggg gct act tta cgg ttc tca tgt gat agt ccc tac tac ttg att ggt 722
Gly Ala Thr Leu Arg Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly
220 225 230 235

caa gaa aca tta acc tgt cag ggt aat ggt cag tgg aat gga cag ata 770
Gln Glu Thr Leu Thr Cys Gln Gly Asn Gly Gln Trp Asn Gly Gln Ile
240 245 250

cca caa tgt aag aac ttg gtc ttc tgt cct gac ctg gat cct gta aac 818
Pro Gln Cys Lys Asn Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn
255 260 265

cat gct gaa cac aag gtt aaa att ggt gtg gaa caa aaa tat ggt cag 866
His Ala Glu His Lys Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln
270 275 280

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aaa gat gta att cca aat tcg ttg act gag act cta cga ggg aaa ggg    1490
Lys Asp Val Ile Pro Asn Ser Leu Thr Glu Thr Leu Arg Gly Lys Gly
                480                 485                 490

tta aca acc acg tgg ata gga ttg cac aga cta gat gct gag aag ccc    1538
Leu Thr Thr Thr Trp Ile Gly Leu His Arg Leu Asp Ala Glu Lys Pro
            495                 500                 505

ttt att tgg gag tta atg gat cgt agt aat gtg gtt ctg aat gat aac    1586
Phe Ile Trp Glu Leu Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn
        510                 515                 520

cta aca ttc tgg gcc tct ggc gaa cct gga aat gaa act aac tgt gta    1634
Leu Thr Phe Trp Ala Ser Gly Glu Pro Gly Asn Glu Thr Asn Cys Val
    525                 530                 535

tat atg gac atc caa gat cag ttg cag tct gtg tgg aaa acc aag tca    1682
Tyr Met Asp Ile Gln Asp Gln Leu Gln Ser Val Trp Lys Thr Lys Ser
540                 545                 550                 555

tgt ttt cag ccc tca agt ttt gct tgc atg atg gat ctg tca gac aga    1730
Cys Phe Gln Pro Ser Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg
                560                 565                 570

aat aaa gcc aaa tgc gat gat cct gga tca ctg gaa aat gga cac gcc    1778
Asn Lys Ala Lys Cys Asp Asp Pro Gly Ser Leu Glu Asn Gly His Ala
            575                 580                 585

aca ctt cat gga caa agt att gat ggg ttc tat gct ggt tct tct ata    1826
Thr Leu His Gly Gln Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile
        590                 595                 600

agg tac agc tgt gag gtt ctc cac tac ctc agt gga act gaa acc gta    1874
Arg Tyr Ser Cys Glu Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val
    605                 610                 615

act tgt aca aca aat ggc aca tgg agt gct cct aaa cct cga tgt atc    1922
Thr Cys Thr Thr Asn Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile
620                 625                 630                 635

aaa gtc atc acc tgc caa aac ccc cct gta cca tca tat ggt tct gtg    1970
Lys Val Ile Thr Cys Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val
                640                 645                 650

gaa atc aaa ccc cca agt cgg aca aac tcg ata agt cgt gtt ggg tca    2018
Glu Ile Lys Pro Pro Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser
            655                 660                 665
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cct ttc ttg agg ttg cca cgg tta ccc ctc cca tta gct aga gca gcc    2066
Pro Phe Leu Arg Leu Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala
            670                 675                 680

aaa cct cct cca aaa cct aga tcc tca caa ccc tct act gtg gac ttg    2114
Lys Pro Pro Pro Lys Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu
        685                 690                 695

gct tct aaa gtt aaa cta cct gaa ggt cat tac cgg gta ggg tct cga    2162
Ala Ser Lys Val Lys Leu Pro Glu Gly His Tyr Arg Val Gly Ser Arg
700                 705                 710                 715

gcc atc tac acg tgc gag tcg aga tac tac gaa cta ctt gga tct caa    2210
Ala Ile Tyr Thr Cys Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln
                720                 725                 730

ggc aga aga tgt gac tct aat gga aac tgg agt ggt cgg cca gcg agc    2258
Gly Arg Arg Cys Asp Ser Asn Gly Asn Trp Ser Gly Arg Pro Ala Ser
            735                 740                 745

tgt att cca gtt tgt gga cgg tca gac tct cct cgt tct cct ttt atc    2306
Cys Ile Pro Val Cys Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile
        750                 755                 760

tgg aat ggg aat tct aca gaa ata ggt cag tgg ccg tgg cag gca gga    2354
Trp Asn Gly Asn Ser Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly
    765                 770                 775

atc tct aga tgg ctt gca gac cac aat atg tgg ttt ctc cag tgt gga    2402
Ile Ser Arg Trp Leu Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly
780                 785                 790                 795

gga tct cta ttg aat gag aaa tgg atc gtc act gct gcc cac tgt gtc    2450
Gly Ser Leu Leu Asn Glu Lys Trp Ile Val Thr Ala Ala His Cys Val
                800                 805                 810

acc tac tct gct act gct gag att att gac ccc aat cag ttt aaa atg    2498
Thr Tyr Ser Ala Thr Ala Glu Ile Ile Asp Pro Asn Gln Phe Lys Met
            815                 820                 825

tat ctg ggc aag tac tac cgt gat gac agt aga gac gat gac tat gta    2546
Tyr Leu Gly Lys Tyr Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val
        830                 835                 840

caa gta aga gag gct ctt gag atc cac gtg aat cct aac tac gac ccc    2594
Gln Val Arg Glu Ala Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro
    845                 850                 855
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ggc aat ctc aac ttt gac ata gcc cta att caa ctg aaa act cct gtt 2642
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860 865 870 875

act ttg aca aca cga gtc caa cca atc tgt ctg cct act gac atc aca 2690
Thr Leu Thr Thr Arg Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr
880 885 890

aca aga gaa cac ttg aag gag gga aca tta gca gtg gtg aca ggt tgg 2738
Thr Arg Glu His Leu Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp
895 900 905

ggt ttg aat gaa aac aac acc tat tca gag acg att caa caa gct gtg 2786
Gly Leu Asn Glu Asn Asn Thr Tyr Ser Glu Thr Ile Gln Gln Ala Val
910 915 920

cta cct gtt gtt gca gcc agc acc tgt gaa gag ggg tac aag gaa gca 2834
Leu Pro Val Val Ala Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala
925 930 935

gac tta cca ctg aca gta aca gag aac atg ttc tgt gca ggt tac aag 2882
Asp Leu Pro Leu Thr Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys
940 945 950 955

aag gga cgt tat gat gcc tgc agt ggg gac agt gga gga cct tta gtg 2930
Lys Gly Arg Tyr Asp Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val
960 965 970

ttt gct gat gat tcc cgt acc gaa agg cgg tgg gtc ttg gaa ggg att 2978
Phe Ala Asp Asp Ser Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile
975 980 985

gtc agc tgg ggc agt ccc agt gga tgt ggc aag gcg aac cag tac ggg 3026
Val Ser Trp Gly Ser Pro Ser Gly Cys Gly Lys Ala Asn Gln Tyr Gly
990 995 1000

ggc ttc act aaa gtt aac gtt ttc ctg tca tgg att agg cag ttc att 3074
Gly Phe Thr Lys Val Asn Val Phe Leu Ser Trp Ile Arg Gln Phe Ile
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Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
        35   40   45

Phe Asn Ile Pro Val Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
    50   55   60

Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys
65   70   75   80

Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu Asp Ser Cys Val Thr
                85   90   95

Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
            100   105   110

Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Ala Cys Arg
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Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu Lys Gly Cys Pro Leu
    130   135   140

Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn
145   150   155   160

Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
                165   170   175

Met Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Asn Phe Pro

180 185 190

Pro Lys Cys Ile Arg Glu Cys Ala Met Val Ser Ser Pro Glu His Gly
195 200 205

Lys Val Asn Ala Leu Ser Gly Asp Met Ile Glu Gly Ala Thr Leu Arg
210 215 220

Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
225 230 235 240

Cys Gln Gly Asn Gly Gln Trp Asn Gly Gln Ile Pro Gln Cys Lys Asn
245 250 255

Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Lys
260 265 270

Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
275 280 285

Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asp
290 295 300

Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser
305 310 315 320

Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val
325 330 335

Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
340 345 350

Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
355 360 365

Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Leu Pro
370 375 380

Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp
385 390 395 400

Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser Glu Glu Leu Lys Ser
405 410 415

Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Arg Ser Ser Thr Ala Gly
420 425 430

Lys Ser Gly Cys Pro Asp Gly Trp Phe Glu Val Asp Glu Asn Cys Val

435 440 445

Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val Cys
450 455 460

Thr Asn Met Ala Ala Arg Leu Ala Val Leu Asp Lys Asp Val Ile Pro
465 470 475 480

Asn Ser Leu Thr Glu Thr Leu Arg Gly Lys Gly Leu Thr Thr Thr Trp
485 490 495

Ile Gly Leu His Arg Leu Asp Ala Glu Lys Pro Phe Ile Trp Glu Leu
500 505 510

Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn Leu Thr Phe Trp Ala
515 520 525

Ser Gly Glu Pro Gly Asn Glu Thr Asn Cys Val Tyr Met Asp Ile Gln
530 535 540

Asp Gln Leu Gln Ser Val Trp Lys Thr Lys Ser Cys Phe Gln Pro Ser
545 550 555 560

Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg Asn Lys Ala Lys Cys
565 570 575

Asp Asp Pro Gly Ser Leu Glu Asn Gly His Ala Thr Leu His Gly Gln
580 585 590

Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile Arg Tyr Ser Cys Glu
595 600 605

Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val Thr Cys Thr Thr Asn
610 615 620

Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile Lys Val Ile Thr Cys
625 630 635 640

Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val Glu Ile Lys Pro Pro
645 650 655

Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser Pro Phe Leu Arg Leu
660 665 670

Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala Lys Pro Pro Pro Lys
675 680 685

Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu Ala Ser Lys Val Lys

690 695 700

Leu Pro Glu Gly His Tyr Arg Val Gly Ser Arg Ala Ile Tyr Thr Cys
705 710 715 720

Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln Gly Arg Arg Cys Asp
725 730 735

Ser Asn Gly Asn Trp Ser Gly Arg Pro Ala Ser Cys Ile Pro Val Cys
740 745 750

Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile Trp Asn Gly Asn Ser
755 760 765

Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly Ile Ser Arg Trp Leu
770 775 780

Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly Gly Ser Leu Leu Asn
785 790 795 800

Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Thr Tyr Ser Ala Thr
805 810 815

Ala Glu Ile Ile Asp Pro Asn Gln Phe Lys Met Tyr Leu Gly Lys Tyr
820 825 830

Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val Gln Val Arg Glu Ala
835 840 845

Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro Gly Asn Leu Asn Phe
850 855 860

Asp Ile Ala Leu Ile Gln Leu Lys Thr Pro Val Thr Leu Thr Thr Arg
865 870 875 880

Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr Thr Arg Glu His Leu
885 890 895

Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp Gly Leu Asn Glu Asn
900 905 910

Asn Thr Tyr Ser Glu Thr Ile Gln Gln Ala Val Leu Pro Val Val Ala
915 920 925

Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala Asp Leu Pro Leu Thr
930 935 940

Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys Lys Gly Arg Tyr Asp

945 950 955 960

Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Phe Ala Asp Asp Ser
965 970 975

Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile Val Ser Trp Gly Ser
980 985 990

Pro Ser Gly Cys Gly Lys Ala Asn Gln Tyr Gly Gly Phe Thr Lys Val
995 1000 1005

Asn Val Phe Leu Ser Trp Ile Arg Gln Phe Ile
1010 1015

What is claimed is:

1. A method for treating gram negative bacterial infection in a subject comprising administering an amount of recombinant Factor C effective for producing bacteriostasis.

2. The method of claim 1, wherein said recombinant Factor C is produced by a yeast host cell or by an insect host cell.

3. The method of claim 1, wherein said recombinant Factor C lacks serine protease activity but retains lipid A binding activity.

4. The method of claim 1, wherein said recombinant Factor C is encoded by a nucleic acid that hybridizes to a nucleic acid having the sequence of SEQ ID NO:1 or SEQ ID NO:3 under stringent conditions.

5. The method of claim 1, wherein the recombinant Factor C has the amino acid sequence of SEQ ID NO:2 or of SEQ ID NO:4, has residues 1-766 of SEQ ID NO:4, residues 29-330 of SEQ ID NO:4, residues 29-201 of SEQ ID NO:4, or residues 264-330 of SEQ ID NO:4, or has three sushi domains linked by random amino acid sequences.

6. The method of claim 5, wherein the recombinant Factor C has at least amino acids 60-70, 170-185, and 270-280 of SEQ ID NO:4.

7. The method of claim 1, wherein the Gram negative bacterial infection comprises bacteria selected from the group consisting of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. typhimurium*.

8. The method of claim 1, wherein the subject is a mammal.

9. The method of claim 1, wherein the amount administered is within the range of 0.1 to 3 mg per kg body weight of the subject.

10. An isolated polypeptide comprising a lipopolysaccharide binding domain of a Factor C protein.

11. The polypeptide of claim 10, wherein said polypeptide is substantially free of hemolytic activity but retains lipid A binding activity.

12. The polypeptide of claim 10, wherein the lipopolysaccharide binding domain of Factor C protein is selected from the group consisting of:
amino acids 1-333 of a Factor C protein;
at least one member selected from the group consisting of a sushi 1 domain of a Factor C protein, a sushi 2 domain of a Factor C protein, and a sushi 3 domain of a Factor C protein;
a sushi-1 peptide; a sushi-1Δ peptide; a sushi-3 peptide; a sushi-3Δ peptide; a sushi-4 peptide; a sushi-5 peptide; a sushi-6-vg1 peptide; a sushi-7-vg2 peptide; a sushi-8-vg3 peptide; and a sushi-9-vg4 peptide.

13. The polypeptide of claim 10, further comprising a secretory signal sequence of a vitellogenin protein.

14. The polypeptide of claim 13 that is purified SSCrFCES.

15. The polypeptide of claim 10, comprising a member selected from the group consisting of a sushi-1 peptide, a sushi-1Δ peptide, a sushi-3 peptide, and a sushi-3Δ peptide.

16. The polypeptide of claim 10, further comprising a reporter protein or an affinity tag.

17. The polypeptide of claim 16, comprising a reporter protein selected from the group consisting of green fluorescent protein (GFP), alkaline phosphatase, a peroxidase, and a luciferase.

18. The polypeptide of claim 17, comprising a member selected from the group consisting of SSCrFC-sushi-1-GFP, SSCrFC-sushi-3-GFP, and SSCrFC-sushi-1,2,3-GFP.

19. The polypeptide of claim 16, comprising an affinity tag selected from the group consisting of polyhistidine or biotin.

20. A method for treating sepsis caused by a gram negative bacterial infection comprising administering a polypeptide of claim 10 to a subject in an amount effective to bind lipopolysaccharide of said gram negative bacteria and ameliorate inflammatory response to said lipopolysaccharide.

21. The method of claim 20, wherein said polypeptide is a member selected from the group consisting of a sushi-1 peptide, a sushi-1Δ peptide, a sushi-3 peptide, a sushi-3Δ peptide, a sushi-4 peptide, a sushi-5 peptide, a sushi-6-vg1 peptide, a sushi-7-vg2 peptide, a sushi-8-vg3 peptide, and a sushi-9-vg4 peptide.

22. The method of claim 20, wherein said polypeptide is substantially free of hemolytic activity but retains lipid A binding activity.

23. The method of claim 20, wherein the gram negative bacterial infection comprises bacteria selected from the group consisting of *P. aeruginosa*, *K. pneumoniae*, and *H. pylori*.

24. The method of claim 20, wherein the subject is a mammal.

25. A pharmaceutical composition comprising a therapeutically effective amount of a polypeptide of claim 10 and a pharmaceutically acceptable carrier for topical formulation.

26. A method for treating or preventing infection of a wound by gram negative bacteria comprising administering the composition of claim 25 to said wound.

27. A method for the detection of gram negative bacteria or of lipopolysaccharide in a sample comprising contacting a sample to be assayed for the presence of said gram negative bacteria or lipopolysaccharide with the polypeptide of claim 12 wherein presence of gram negative bacteria or lipopolysaccharide is indicated by a complex between said gram negative bacteria or lipopolysaccharide and said polypeptide of claim 12.

28. The method of claim 27, comprising an *in situ* histologic assay.

29. The method of claim 27, which is a solution assay.

30. The method of claim 27, wherein said polypeptide is immobilized.

31. The method of claim 27, wherein gram negative bacteria or lipopolysaccharide of said sample is immobilized.

32. A method for the detection of whole or fragmentary gram negative bacteria or of lipopolysaccharide in a sample comprising contacting a sample to be assayed for the presence thereof with a polypeptide of claim 10, further comprising a reporter protein.

33. The method of claim 32, wherein said reporter protein comprises a green fluorescent protein.

34. The method of claim 33, wherein said sample comprises tissues or cells and said polypeptide comprises a member selected from the group consisting of SSCrFC-sushi-1-GFP, SSCrFC-sushi-3-GFP, and SSCrFC-sushi-1,2,3-GFP.

35. A method for preserving a sample from contamination by gram negative bacteria comprising adding a polypeptide of claim 10 to said sample in an amount effective for preventing the growth of said gram negative bacteria.

36. A method for purifying a sample by removal of endotoxin comprising immobilizing a polypeptide of claim 12 on an insoluble substrate, contacting said sample with the immobilized polypeptide, and separating said sample from said immobilized polypeptide.

37. An isolated nucleic acid encoding the polypeptide of claim 10.

38. An isolated nucleic acid comprising a nucleic acid encoding a lipopolysaccharide binding portion of a Factor C protein of a horseshoe crab selected from the group consisting of:

amino acids 1-333 of a Factor C protein;

at least one member selected from the group consisting of a sushi 1 domain of a Factor C protein, a sushi 2 domain of a Factor C protein, and a sushi 3 domain of a Factor C protein;

a sushi-1 peptide; a sushi-1Δ peptide; a sushi-3 peptide; a sushi-3Δ peptide; a sushi-4 peptide; a sushi-5 peptide; a sushi-6-vg1 peptide; a sushi-7-vg2 peptide; a sushi-8-vg3 peptide; and a sushi-9-vg4 peptide.

39. The isolated nucleic acid of claim 38, further comprising a nucleic acid encoding a secretion signal sequence of a vitellogenin protein.

40. The isolated nucleic acid of claim 38, further comprising a nucleic acid that encodes a reporter protein or an affinity tag fused to the nucleic acid encoding the lipopolysaccharide binding portion of a Factor C protein.

41. The isolated nucleic acid of claim 40, comprising a nucleic acid encoding a reporter protein selected from the group consisting of green fluorescent protein, alkaline phosphatase, a peroxidase, and a luciferase.

42. The isolated nucleic acid of claim 40, comprising a nucleic acid encoding an affinity tag selected from the group consisting of a polyhistidine sequence or biotin.

43. The isolated nucleic acid of claim 39, wherein the nucleic acid encodes a member selected from the group consisting of SSCrFCES, SSCrFC-sushi-1-GFP, SSCrFC-sushi-3-GFP, and SSCrFC-sushi-1,2,3-GFP.

44. A method for producing an isolated lipopolysaccharide binding protein comprising:

i) culturing a host cell transformed with the isolated nucleic acid of claim 38 to produce said lipopolysaccharide binding protein in a culture medium; and

ii) isolating said lipopolysaccharide binding protein from said culture medium.

45. The method of claim 44, wherein said isolating step ii) comprises ultrafiltering the culture medium with 100 kDa and 10 kDa molecular weight cutoff membranes and preparative isoelectric focussing.

46. A recombinant polypeptide produced by the process of claim 44.

47. The method of claim 7, wherein the Gram negative bacterial infection comprises bacteria selected from the group consisting of K. pneumoniae and E. coli.

48. The polypeptide of claim 10, wherein said polypeptide is substantially free of serine protease activity.

49. The method of claim 44, wherein said isolating step ii) comprises ultrafiltering the culture medium with a 10 kDa cutoff membrane and affinity chromatography.

50. The polypeptide of claim 48, further comprising a secretory signal sequence of a vitellogenin protein.

51. The polypeptide of claim 48, further comprising a reporter protein or an affinity tag.

52. The isolated nucleic acid of claim 39, further comprising a nucleic acid that encodes a reporter protein or an affinity tag fused to the nucleic acid encoding the lipopolysaccharide binding portion of a Factor C protein.

Figure 1A

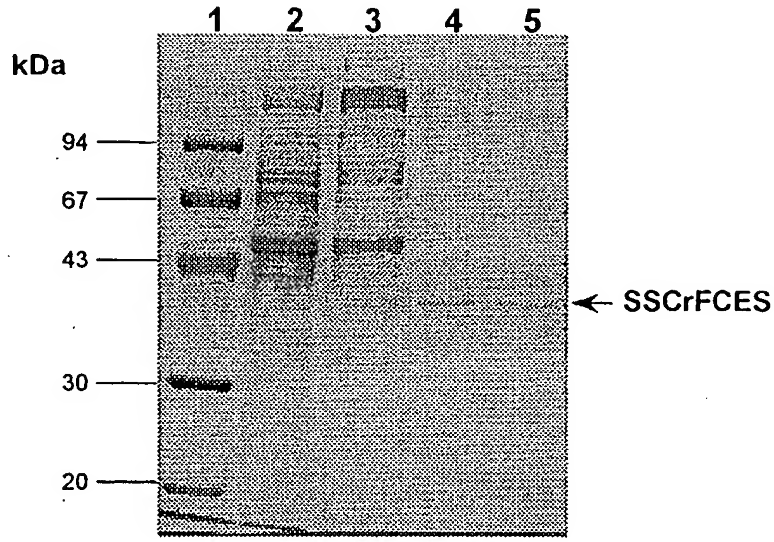


Figure 1B

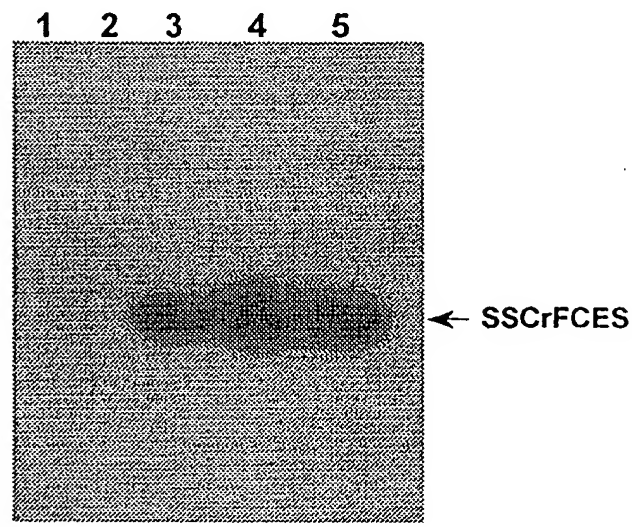


Figure 2A

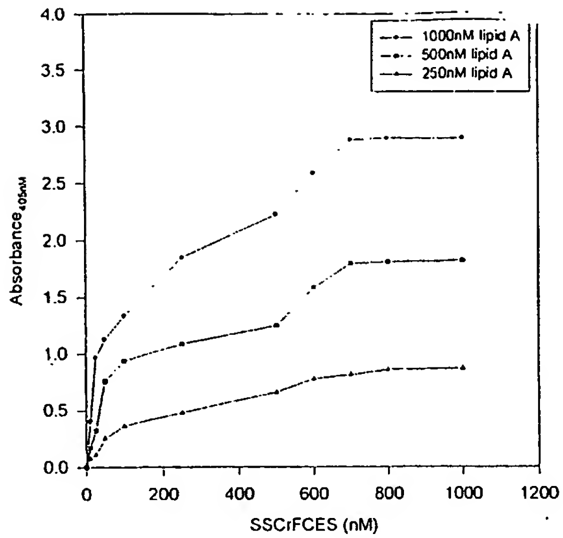


Figure 2B

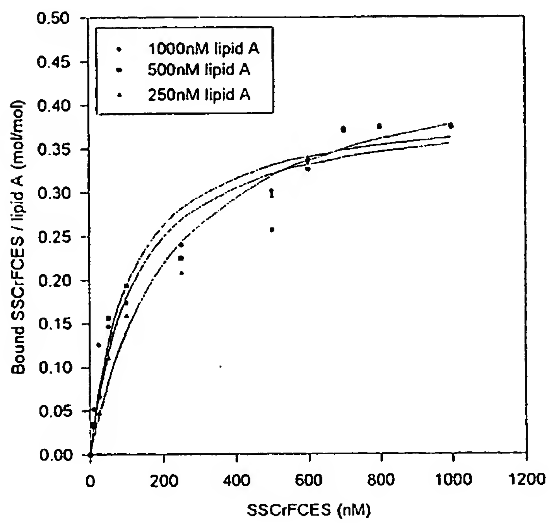


Figure 2C

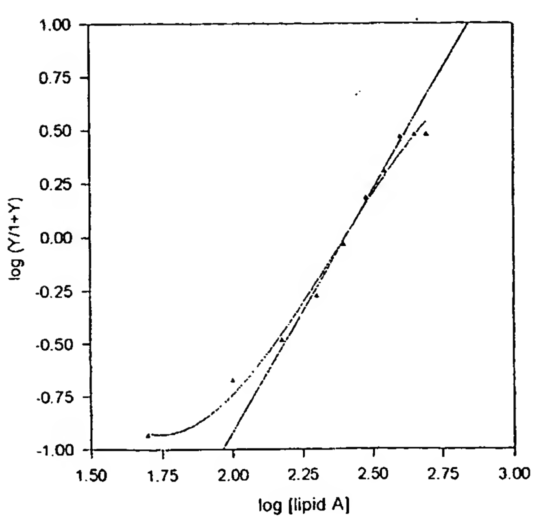


Figure 3A

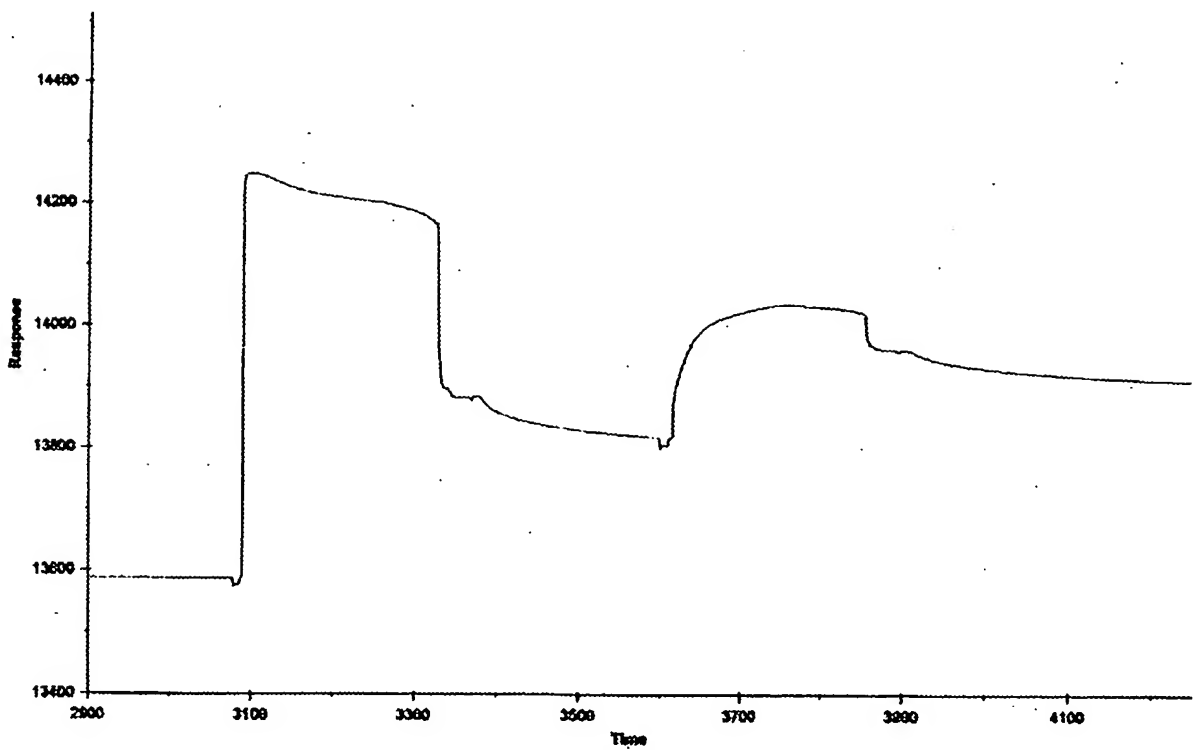

14400
14200
14000
13800
13600
13400
Response
2900
3100
3300
3500
3700
3900
4100
Time

Figure 4A

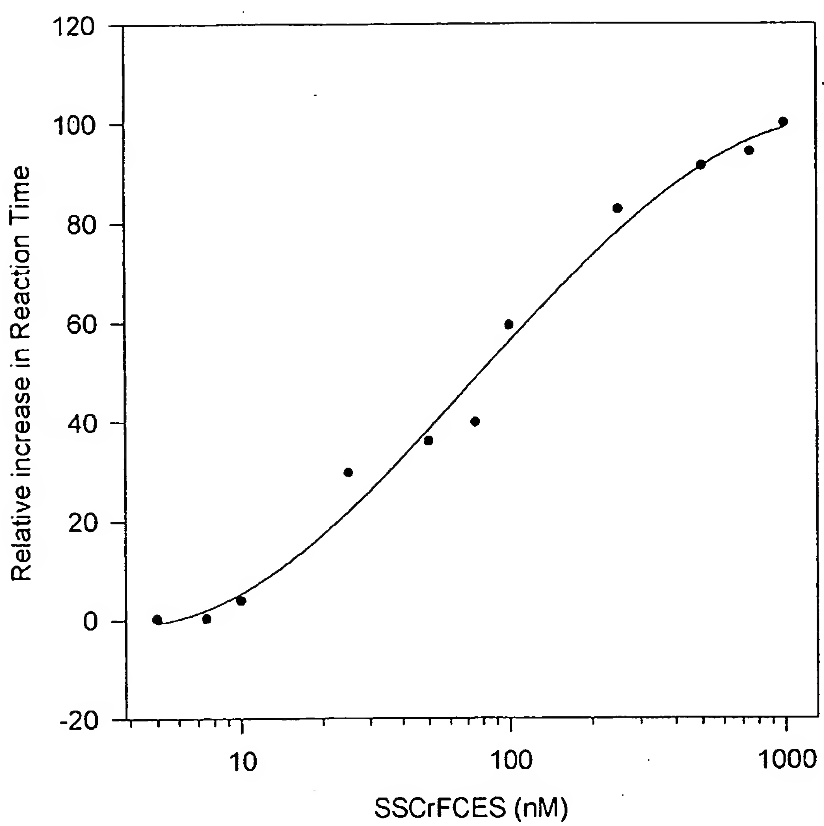

120
100
80
60
40
20
0
-20
Relative increase in Reaction Time
10
100
1000
SSCrFCES (nM)

Figure 5A

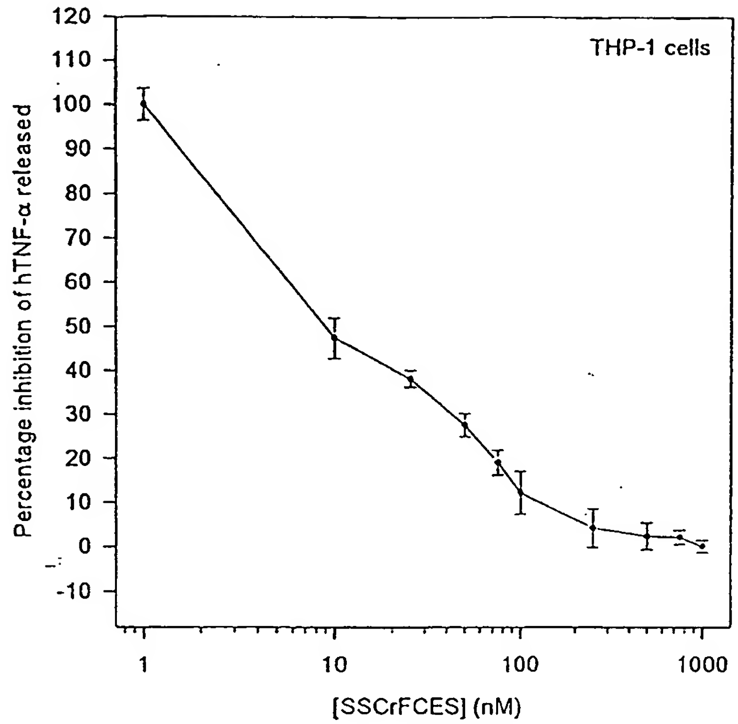


Figure 5B

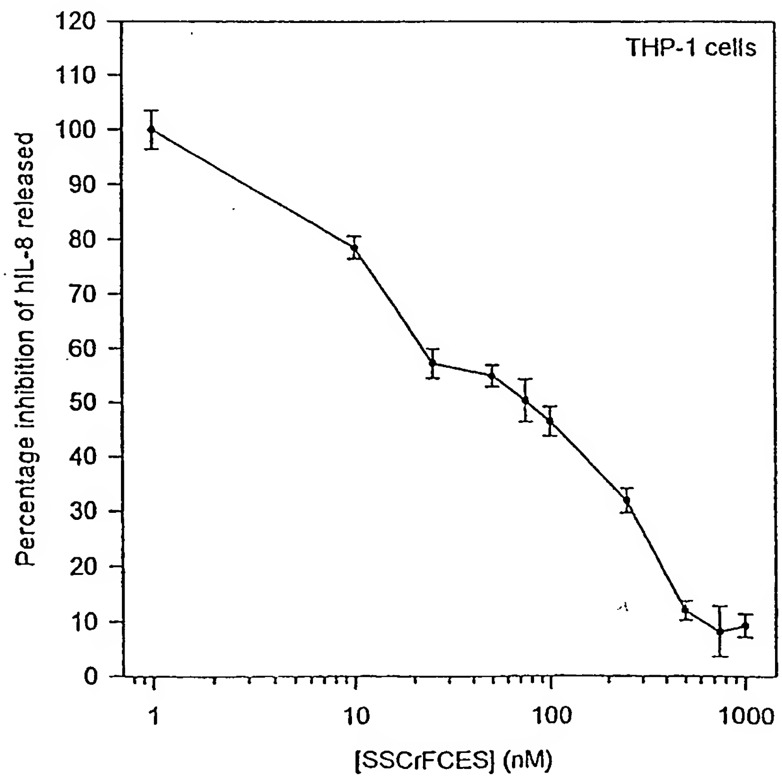


Figure 6A

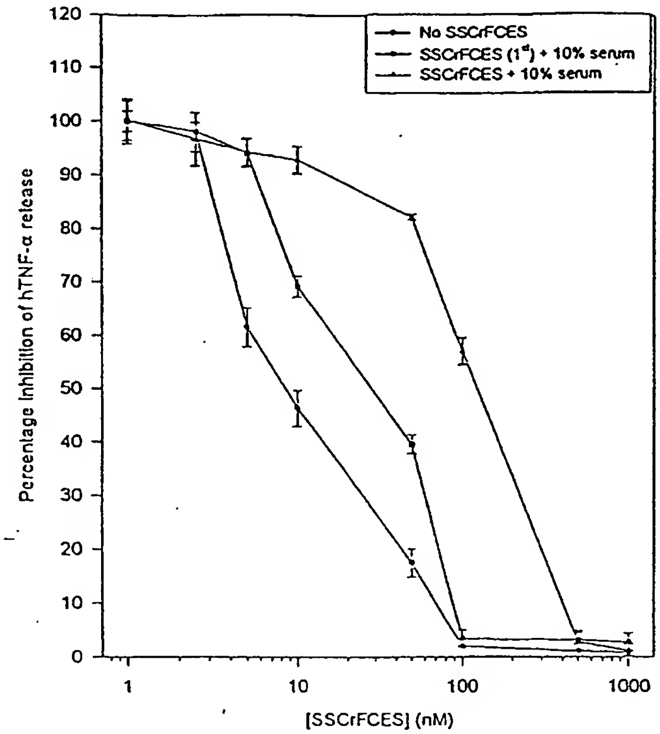


Figure 6B

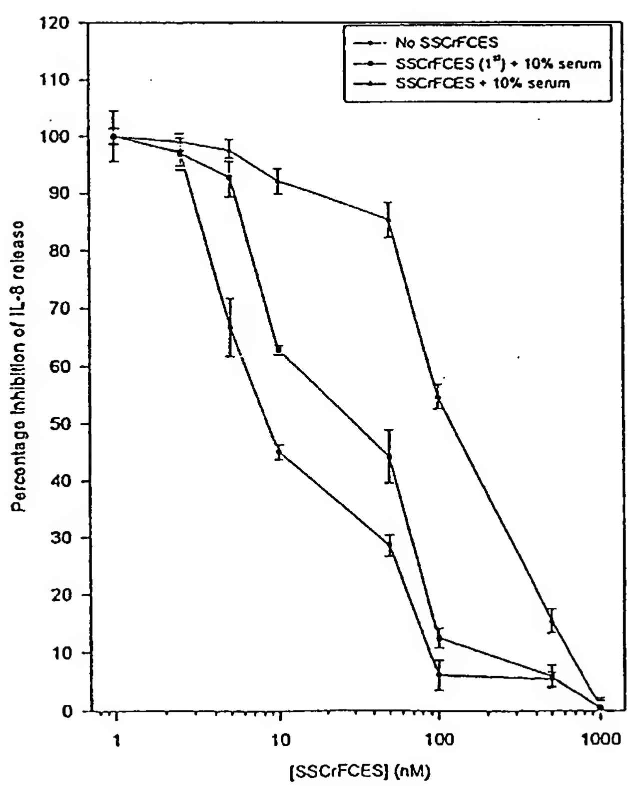


Figure 6C

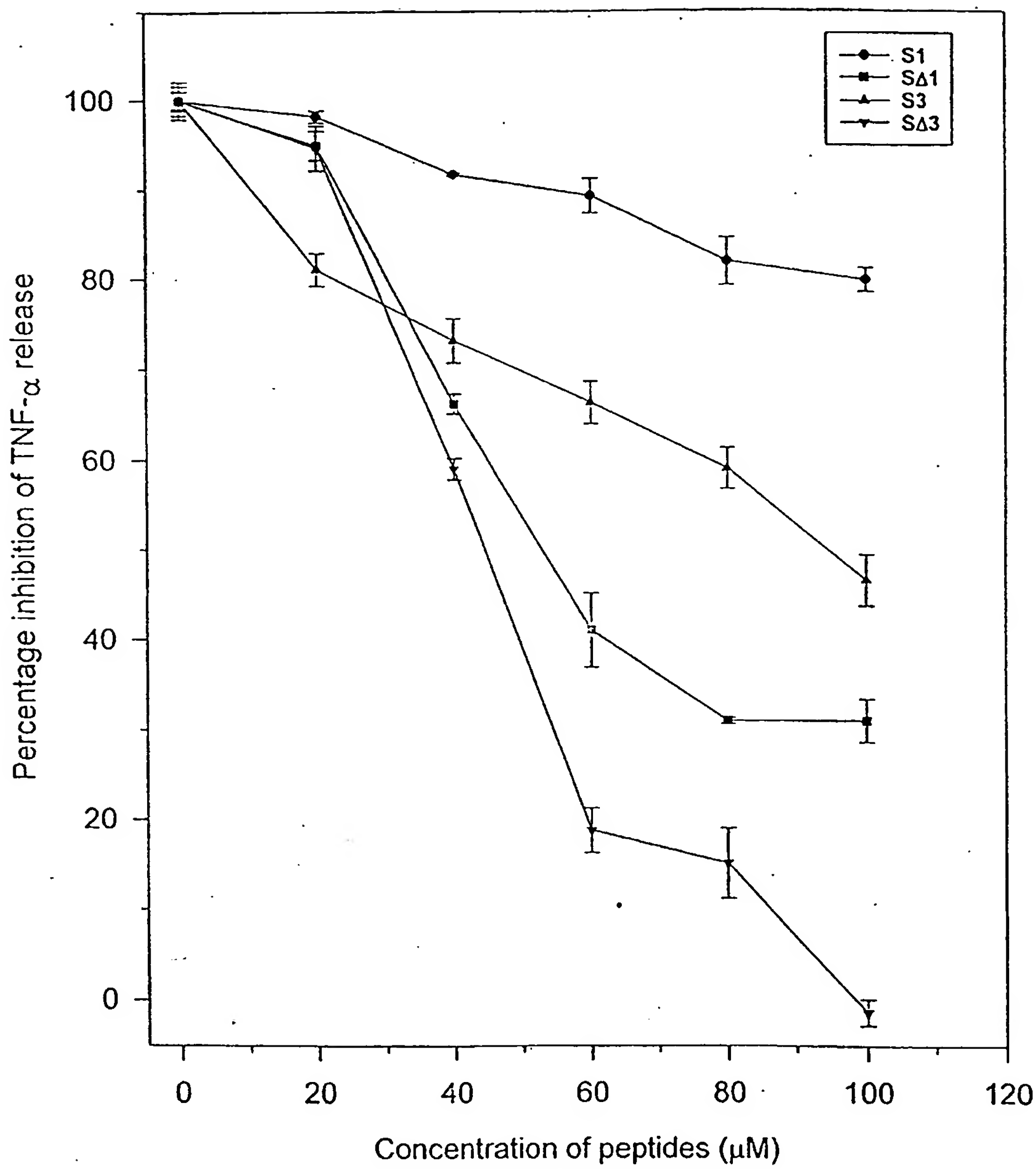

S1
SΔ1
S3
SΔ3
Percentage inhibition of TNF-α release
0
20
40
60
80
100
120
Concentration of peptides (μM)

Figure 7

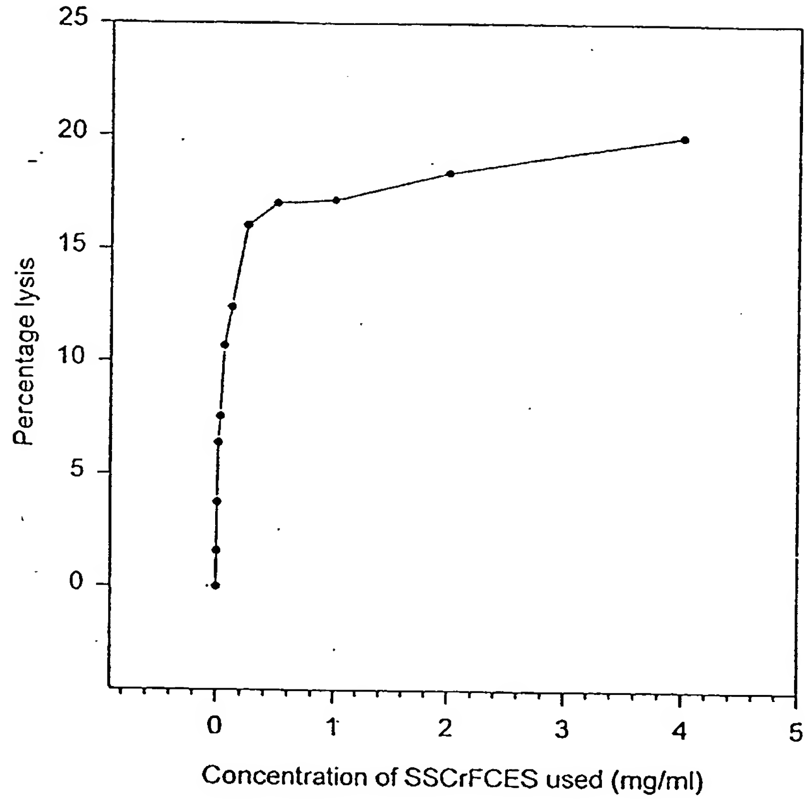

25
20
15
10
5
0
Percentage lysis
0
1
2
3
4
5
Concentration of SSCrFCES used (mg/ml)

Figure 8

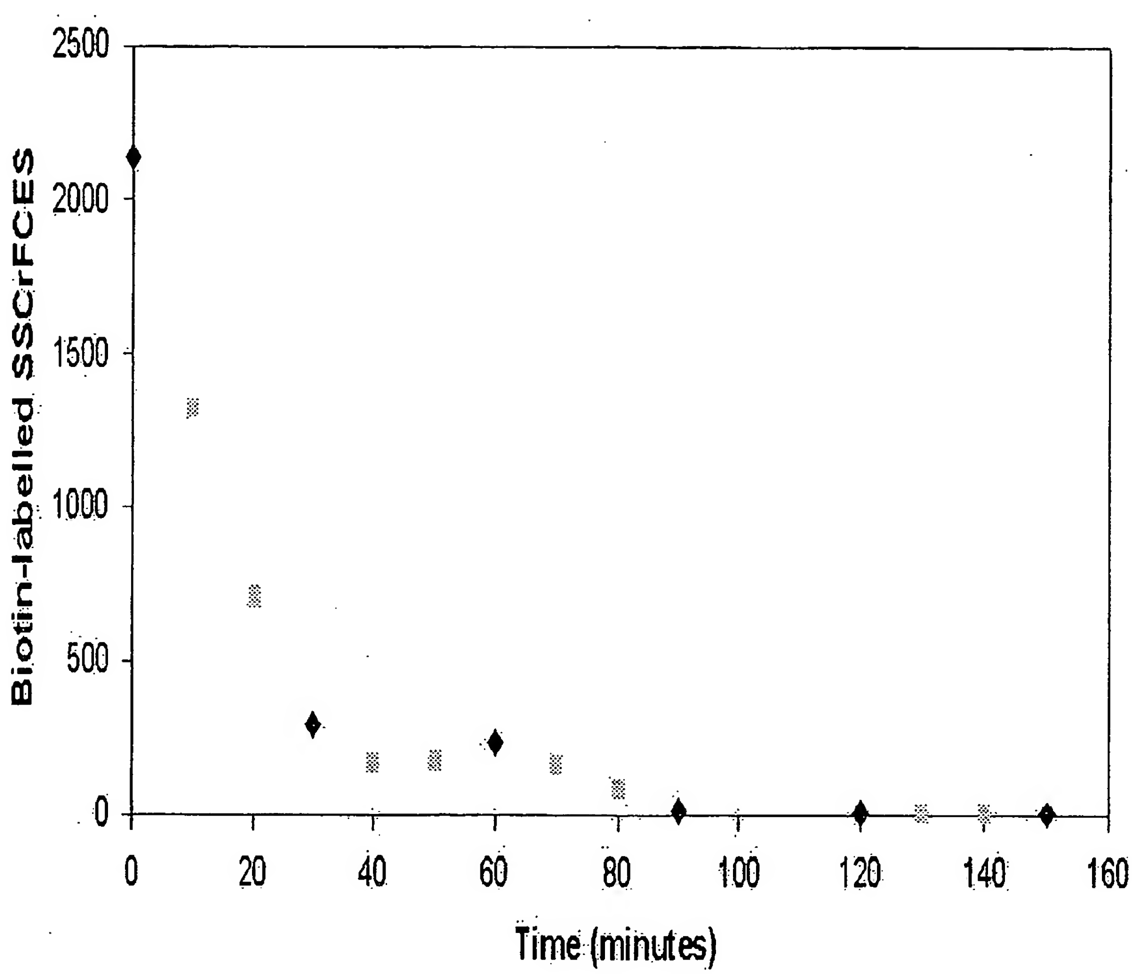

2500
2000
1500
1000
500
0
Biotin-labelled SSCrFCES
0
20
40
60
80
100
120
140
160
Time (minutes)

Figure 9A

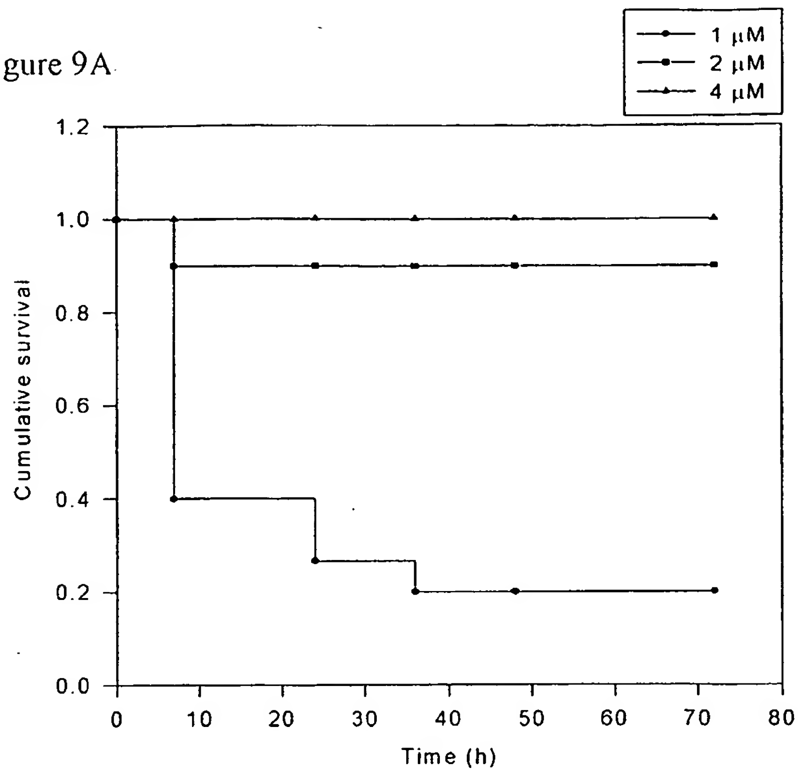


Figure 9B

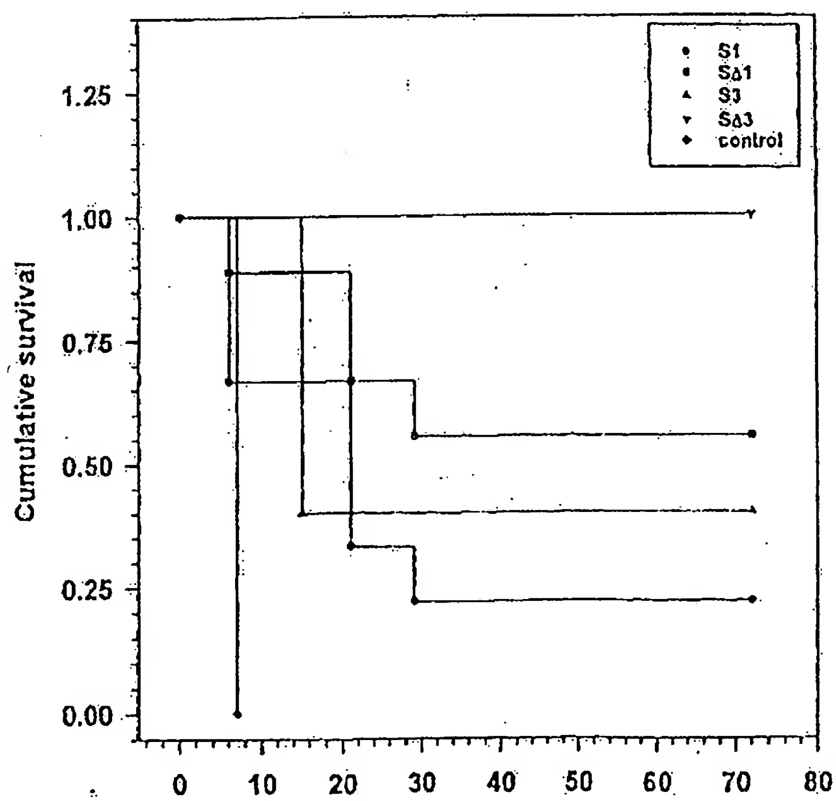


Fig 10A

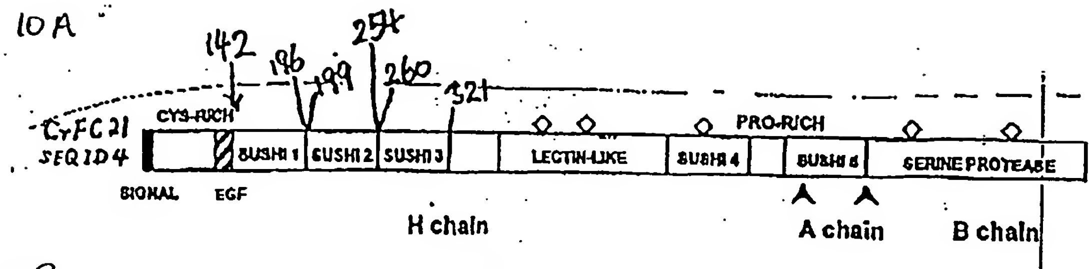
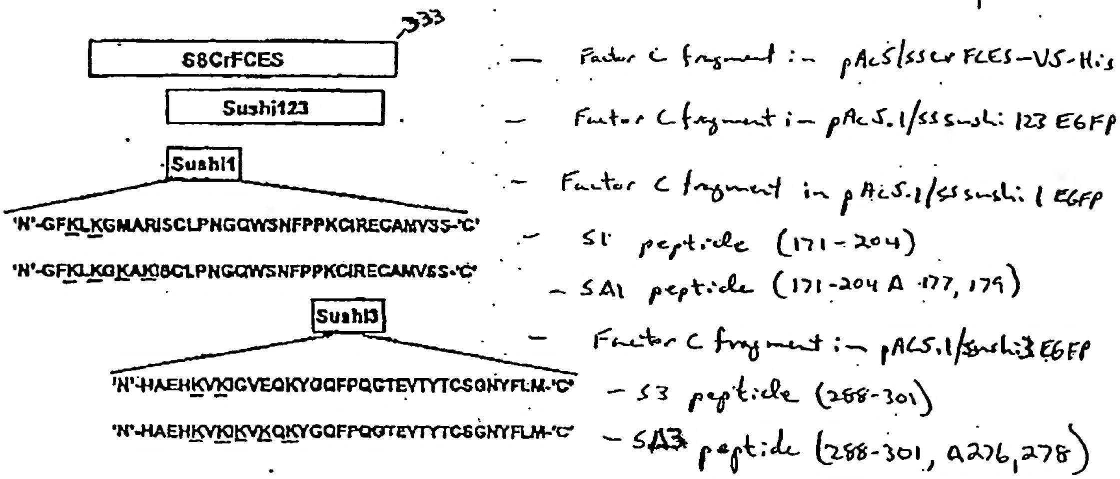


Fig 10B



BHBHB BHBHB

V1: Acetyl-C-VKVKVKV-GSG-VKVKVKV-C-NH2

BH?HB BHPHB

V2: Acetyl-C-VKV<u>S</u>VKV-GSG-VKV<u>S</u>VKV-C-NH2

Fig. 11A

S1	GFKLKGMARISCLPNGQWSNFPPKCIRECAMVSS	(S171-204)
S1△	GFKLKG<u>K</u>A<u>K</u>ISCLPNGQWSNFPPKCIRECAMVSS	(S171-204@177,179)
S3	HAEHKVKIGVEQKYGQFPQGTEVTYTCSGNYFLM	(S268-301)
S3△	HAEHKVKI<u>K</u>V<u>K</u>QKYGQFPQGTEVTYTCSGNYFLM	(<u>S268-301@276,278</u>)
S4	RAEHKVKKIVKQLYGQFRQLTRVTRTCSRFLRRM	
S5	HKVKKIVKQLYRAEHKVKKIVKQL	
S6-vg1	MRKLVLALAKALAKVDKKNL	
S7-vg2	LLNAVPHKATHAALKFLKEK	
S8-vg3	GVSTTVLNIYRGIINLLQLNVKK	
S9-vg4	IYRGIINLIQLAVKKAQNVYQM	

Fig. 11B

Figure 12

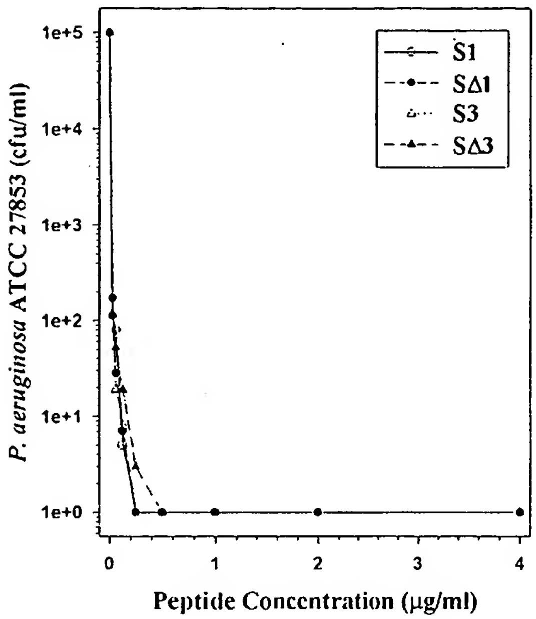


Figure 13

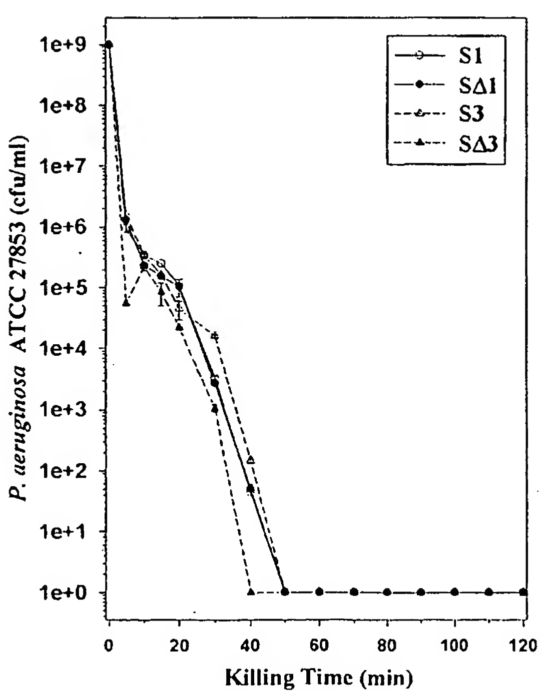


Figure 14

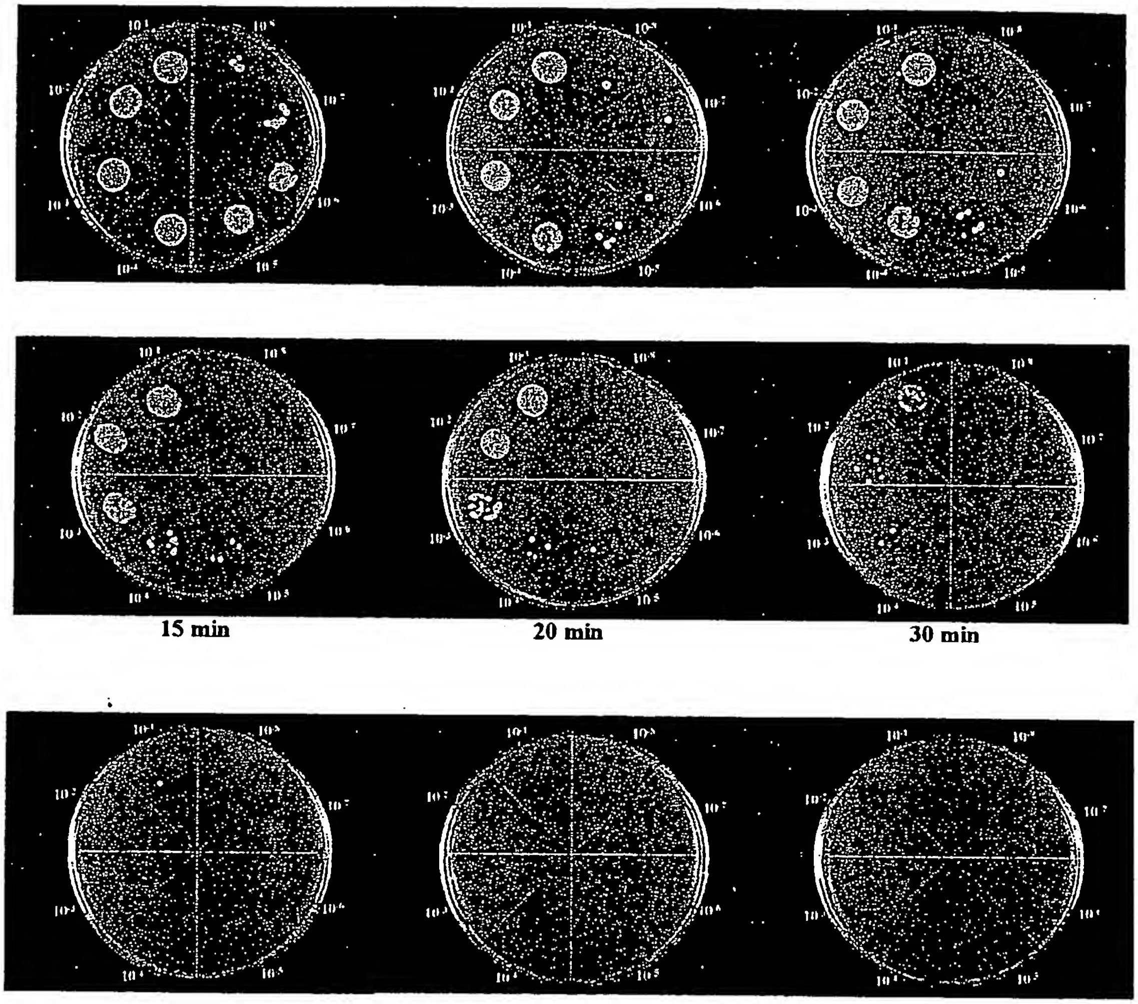
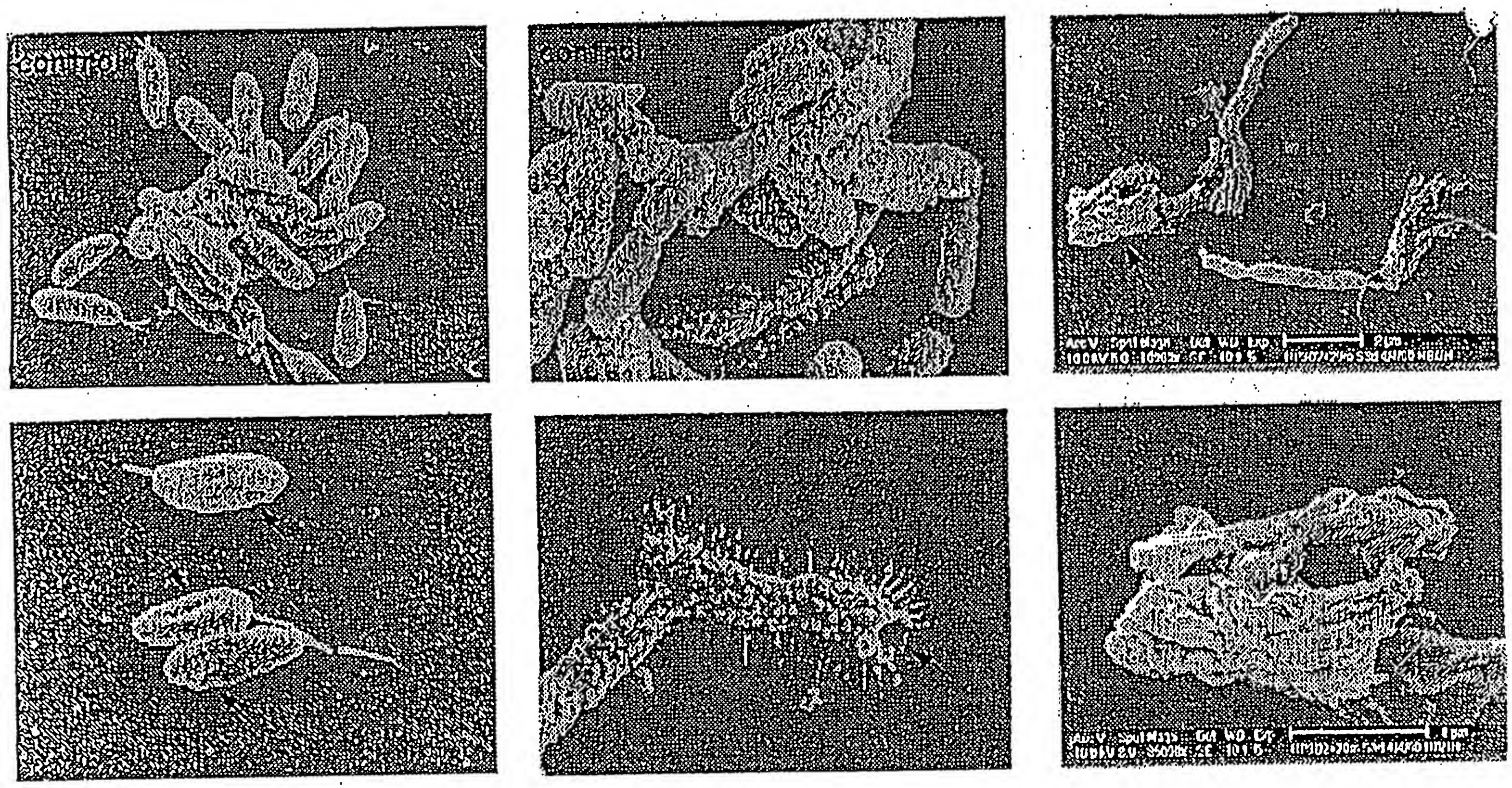


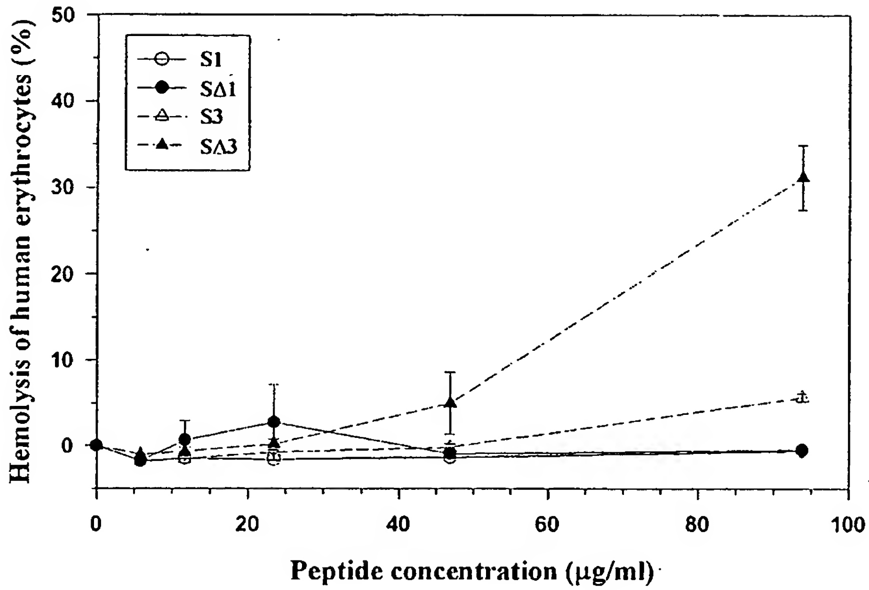
Figure 15 **Scanning EM to show how Sushi peptides kill Bacteria**

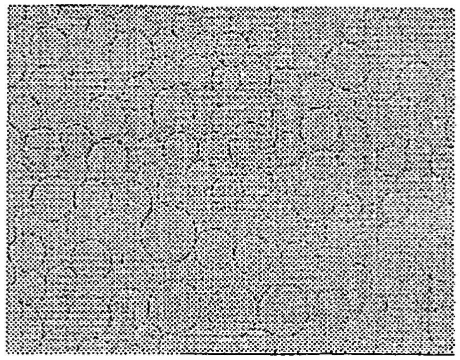


P. aeruginosa *K. pneumoniae* *H. pylori*

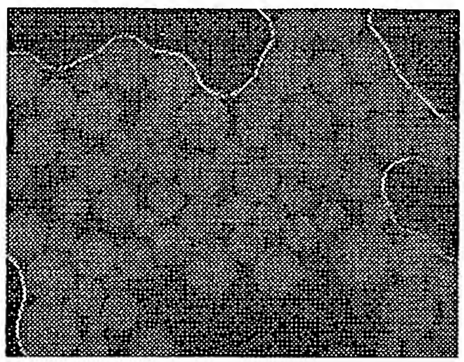
Sushi peptides puncture holes (*P. aeruginosa* & *K. pneumoniae*) into or "de-coat" (*H. pylori*) these multiple antibiotic-resistant strains of bacteria.

Figure 16

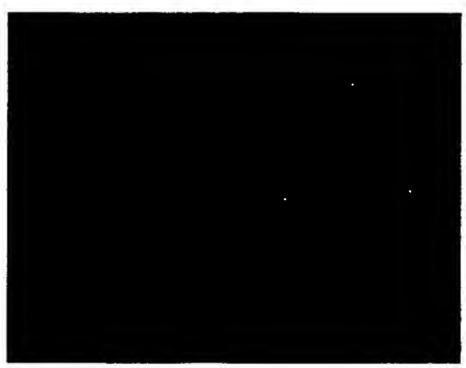

S1
SΔ1
S3
SΔ3
Hemolysis of human erythrocytes (%)
Peptide concentration (µg/ml)
50
40
30
20
10
0
0
20
40
60
80
100



(A)

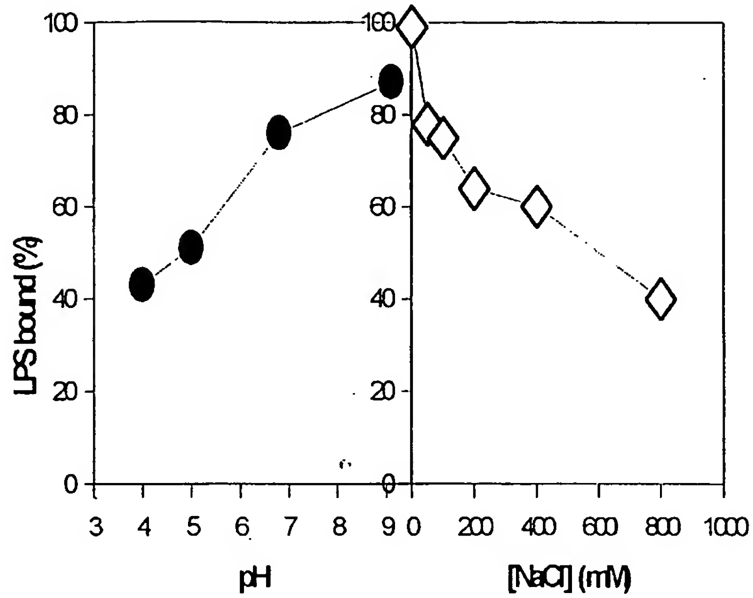


(B)



(C)

Figure 17. An example of FITC-LPS bound to SΔ3-peptide coupled Agarose CL-6B beads viewed under microscope. (A) Bright field observation. (B) UV light fluorescence microsopic view. (C) Beads treated with 1% DOC and observed under UV light - negligible FITC-LPS remained on the bead.



A. Binding efficiency of LPS to the affinity beads under different pH conditions.

B. binding efficiency of LPS to the affinity beads under different ionic strength.

Figure 18. Test of binding of LPS to the peptide affinity beads under different conditions. (A) Different pH: pH 4.0, 5.0 (20 mM sodium acetate), pH 6.8 and pH 9.1 (20 mM Tris-HCl). All buffers were supplemented with 50 mM NaCl. (B) Different ionic strength: 20 mM Tris-HCl (pH 6.8) were supplemented with different concentrations of NaCl, except for the 0 mM point which is in pyrogen-free water as control.

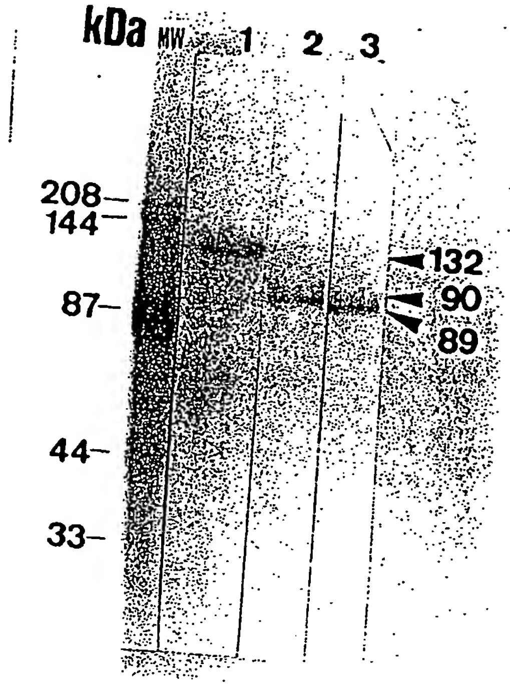


Figure 19

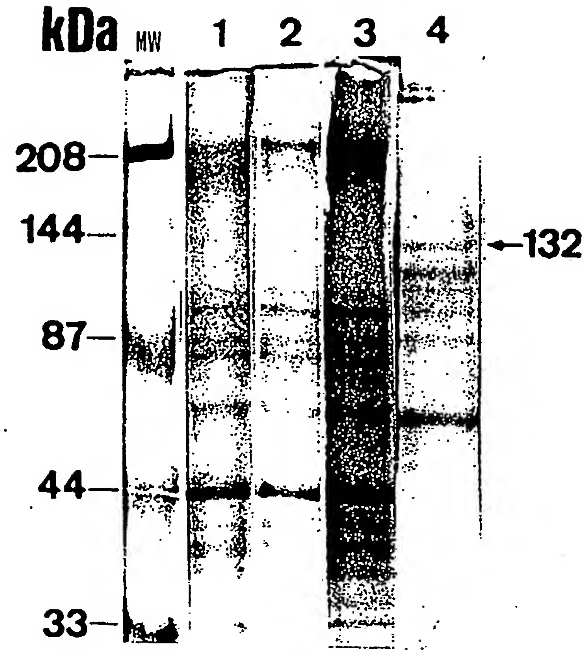


Figure 20

Figure 21 (A) LPS Strips

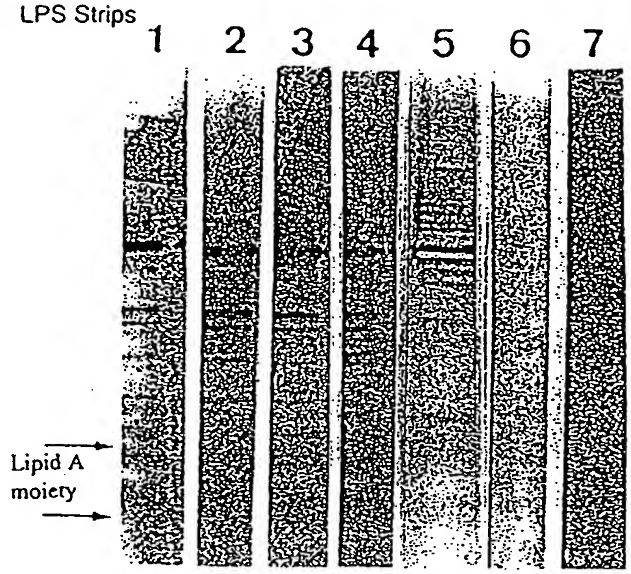


Figure 21 (B) Lipid A strips

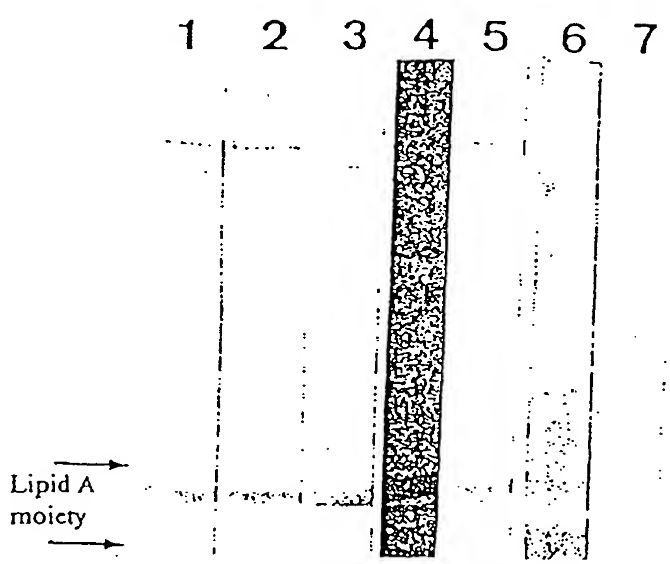


Fig. 22A Crude rFC

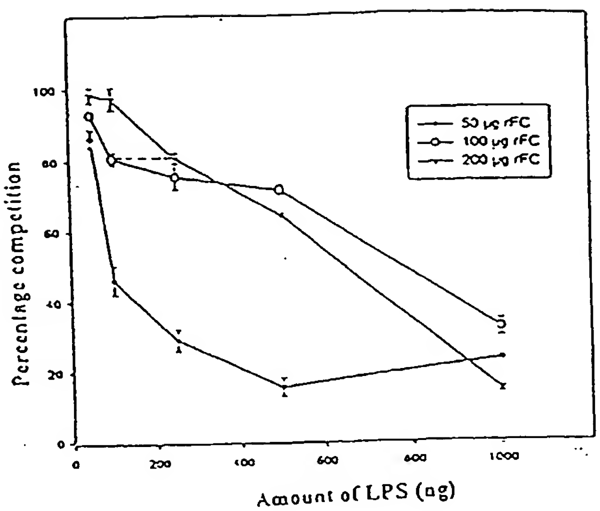
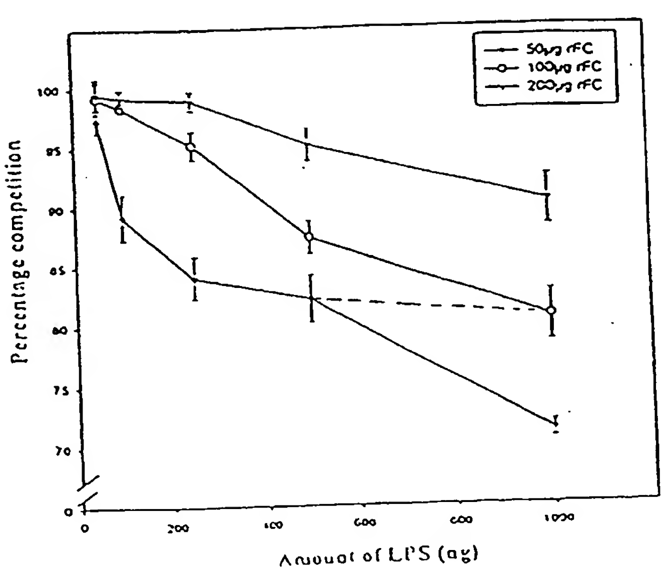


Fig. 22B Biomax-50 enriched rFC



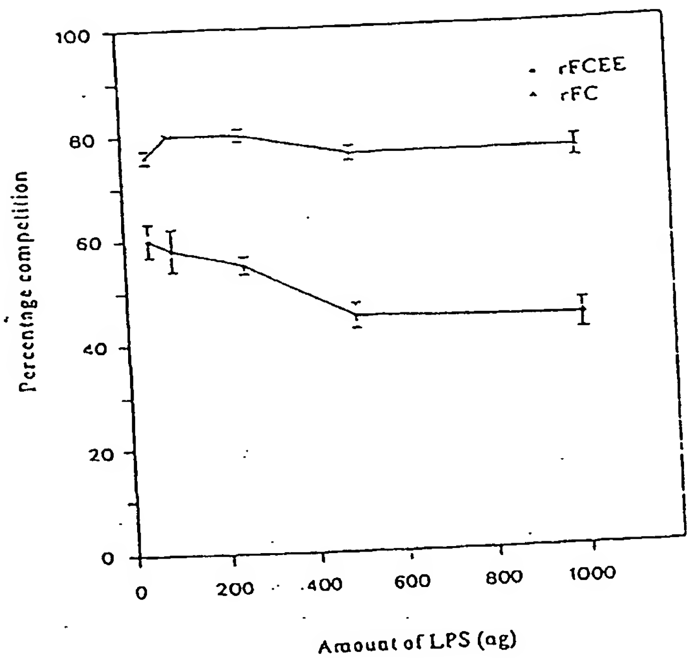

rFCEE
rFC
Percentage competition
100
80
60
40
20
0
0
200
400
600
800
1000
Amount of LPS (ng)

Fig. 23

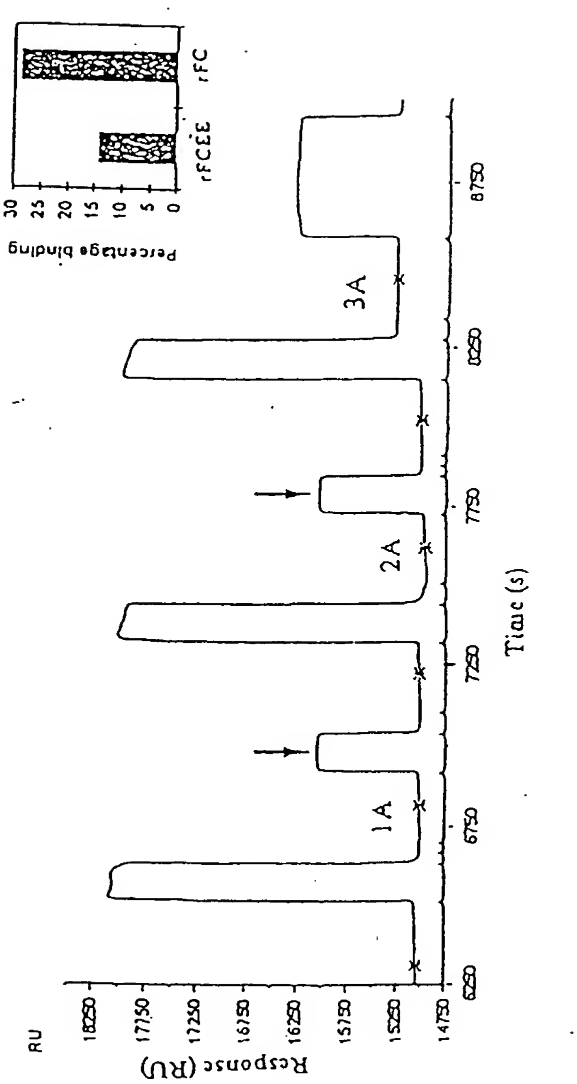

Percentage binding
30
25
20
15
10
5
0
rFCEE
rFC
RU
Response (RU)
18250
17750
17250
16750
16250
15750
15250
14750
1A
2A
3A
6250
6750
7250
7750
8250
8750
Time (s)

Fig. 24

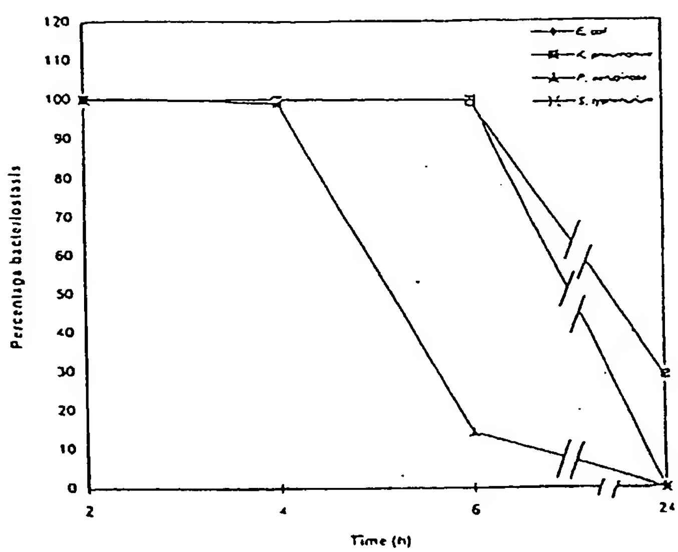

120
110
100
90
80
70
60
50
40
30
20
10
0
Percentage bacteriostasis
2
4
6
24
Time (h)

Fig. 25

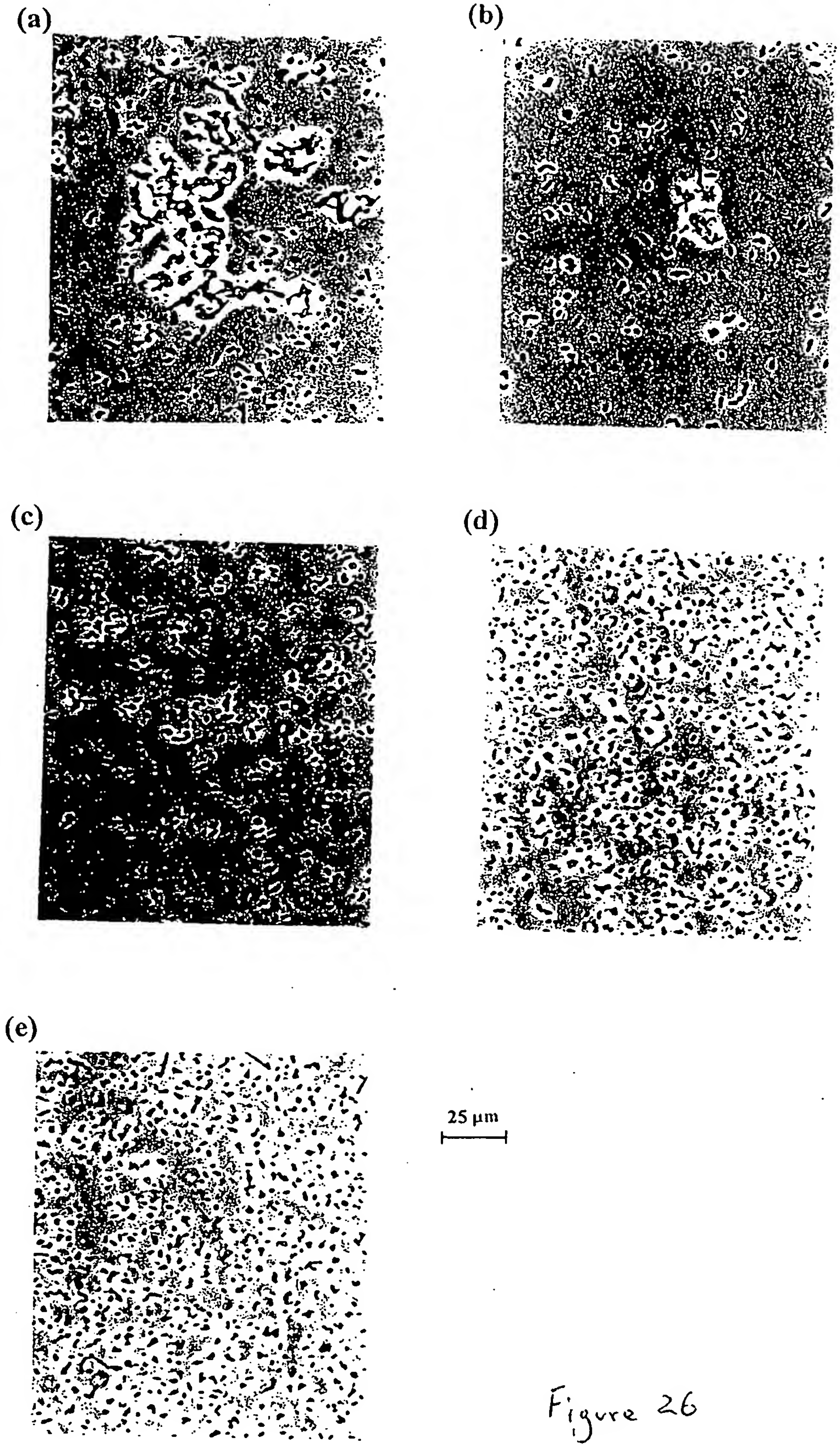


Figure 26

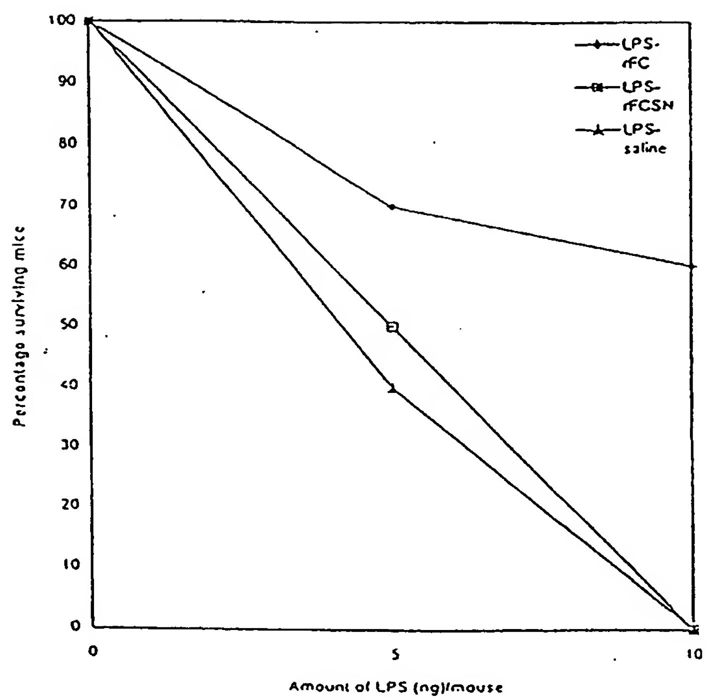

100
90
80
70
60
50
40
30
20
10
0
Percentage surviving mice
0
5
10
Amount of LPS (ng)/mouse
LPS-rFC
LPS-rFCSN
LPS-saline

Fig. 27

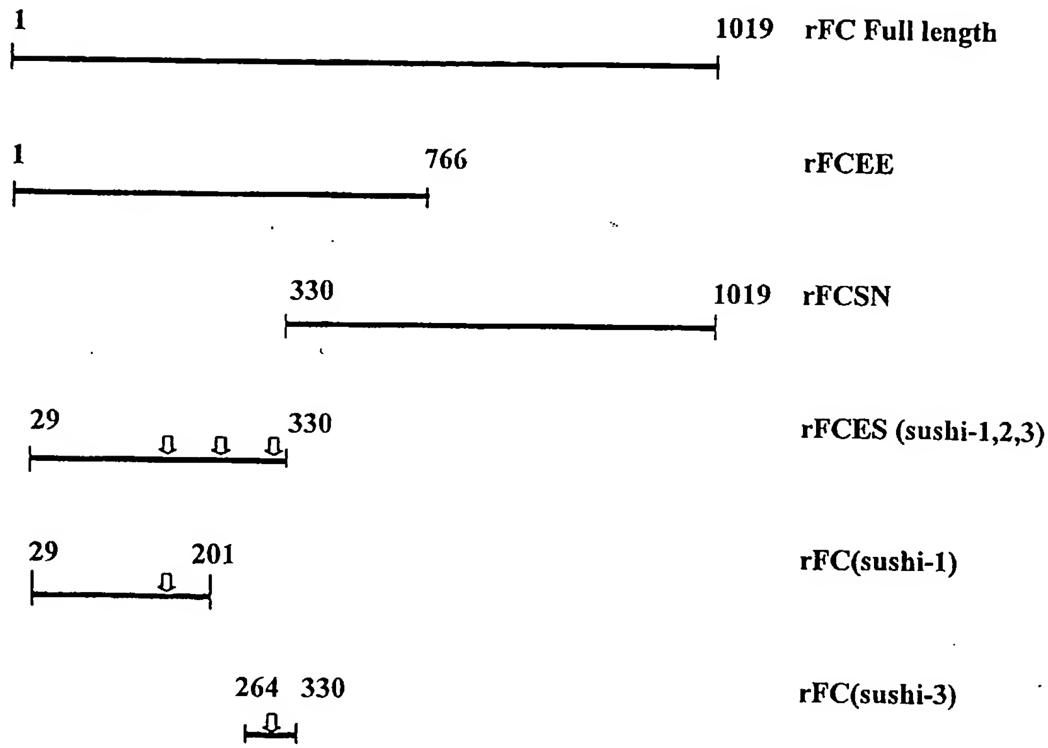

1
1019
rFC Full length
1
766
rFCEE
330
1019
rFCSN
29
330
rFCES (sushi-1,2,3)
29
201
rFC(sushi-1)
264
330
rFC(sushi-3)

Figure 28

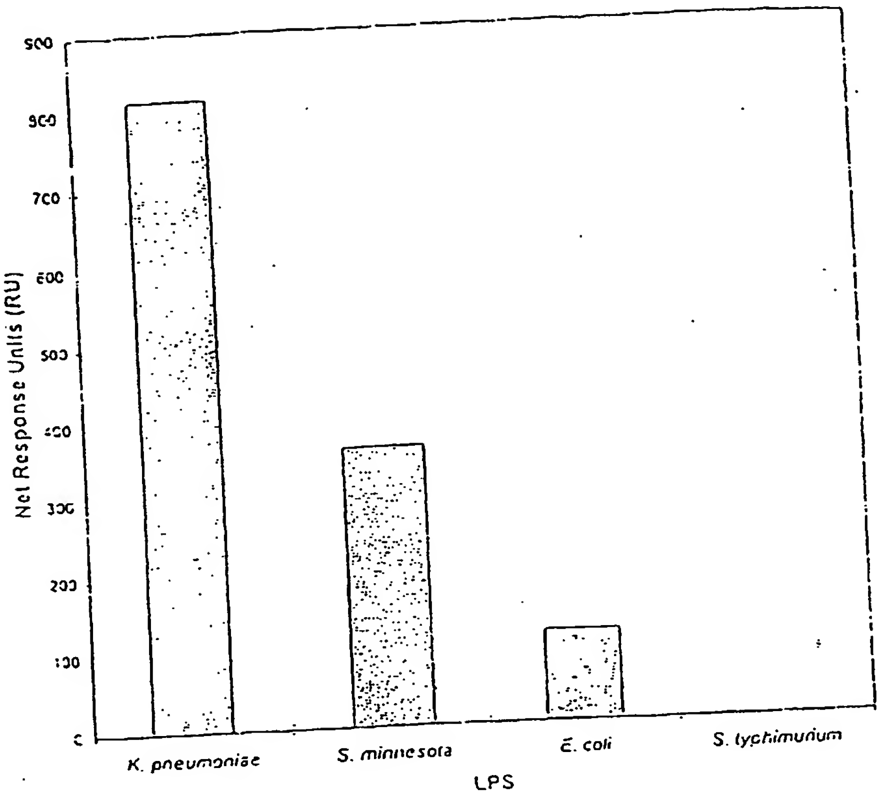

Net Response Units (RU)
700
600
500
400
300
200
100
K. pneumoniae
S. minnesota
E. coli
S. typhimurium
LPS

Fig. 29

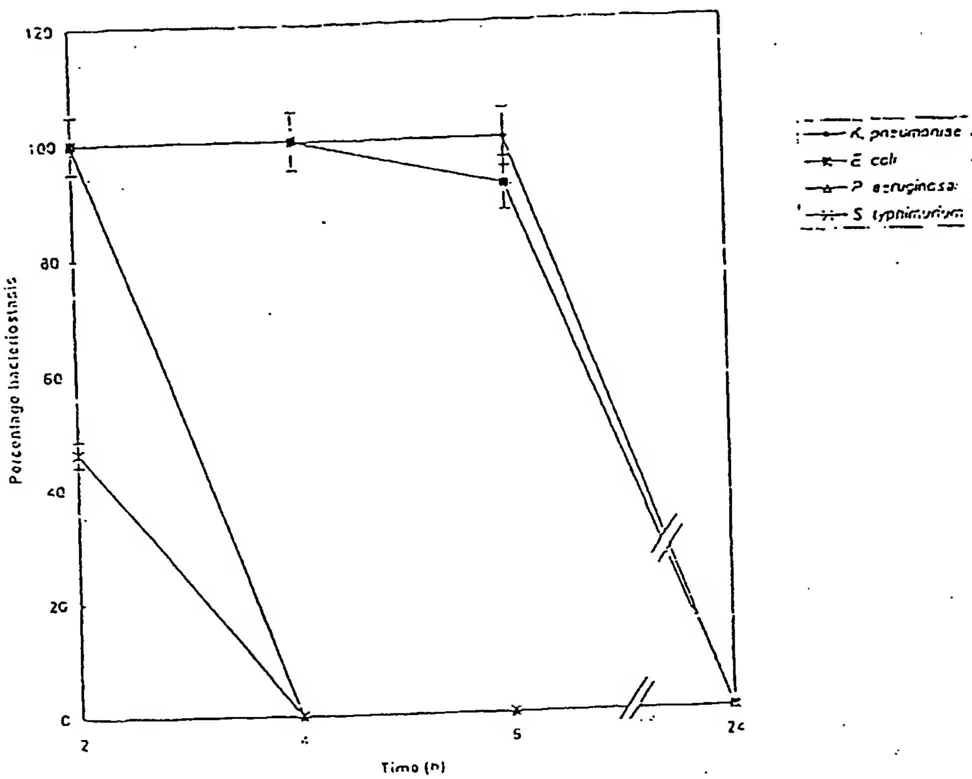

120
100
80
60
40
20
0
Percentage bacteriostasis
2
4
5
24
Time (h)
K. pneumoniae
E. coli
P. aeruginosa
S. typhimurium

Fig. 30

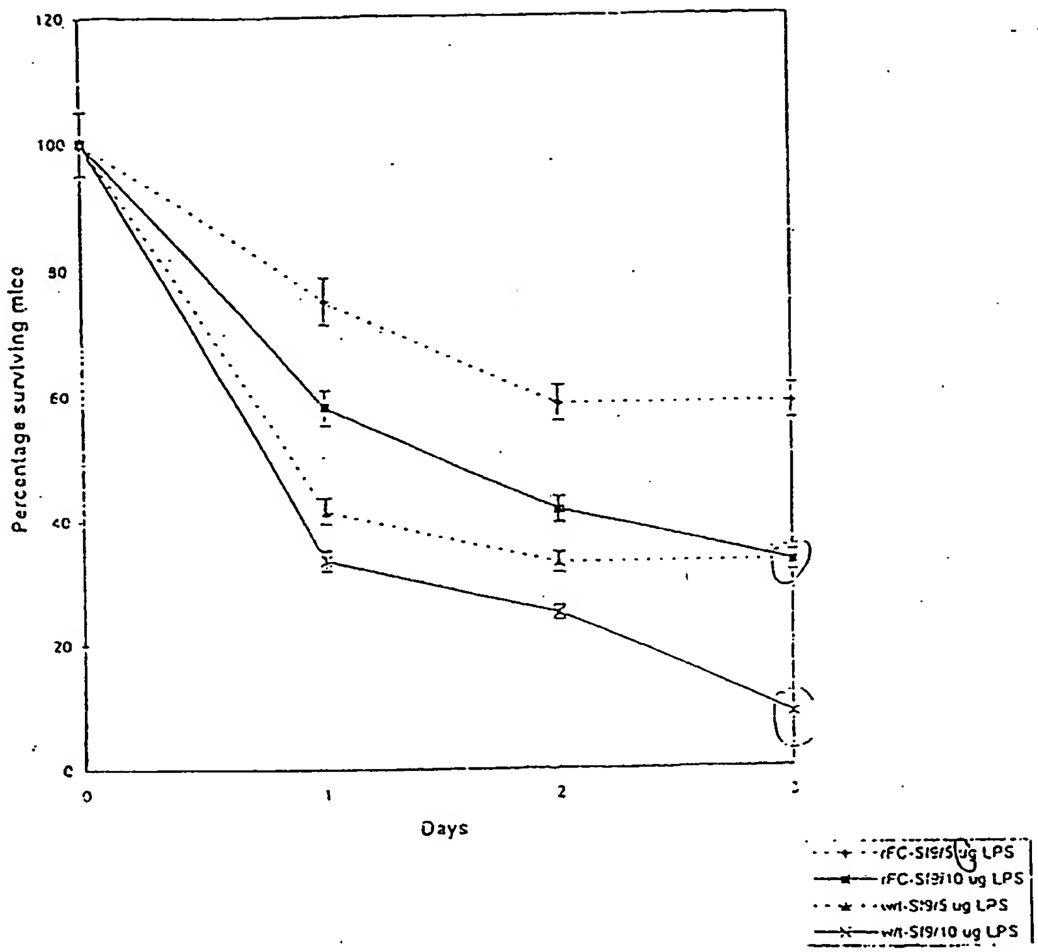

120
100
80
60
40
20
0
Percentage surviving mice
0
1
2
3
Days
rFC-S19/5 ug LPS
rFC-S19/10 ug LPS
wt-S19/5 ug LPS
wt-S19/10 ug LPS

Fig. 31